CHARACTERIZATION OF TICK FEEDING SITES ON SHEEP EXPERIMENTALLY INFECTED WITH THE HUMAN NY-18 ISOLATE OF *ANAPLASMA PHAGOCYTOPHILUM*

By

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OF *ANAPLASMA PHAGOCYTOPHILUM*

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Abstract:

Anaplasma phagocytophilum, first identified as a pathogen of ruminants in Europe, has more recently been recognized as an emerging tick-borne pathogen of humans in the U.S. and Europe. *A. phagocytophilum* is transmitted primarily by ticks of the genus *Ixodes*. Our laboratory recently developed a sheep model for study of the host/ tick/pathogen interactions of the human NY-18 isolate of *A. phagocytophilum.* In this model, sheep became infected with the pathogen within 14 days after inoculation but did not exhibit clinical signs and infected morulae were rarely seen in stained blood smears. However, when ticks were allowed to feed on the infected sheep, they readily acquired *A. phagocytophilum* infection, and 80% to 100% of the tick salivary glands and guts were confirmed by PCR to be infected after a 2- to 4-day feeding period. In this research we examined tick feeding sites to determine the source of *A. phagocytophilum* infection for the ticks using PCR and immunohistochemistry (IHC). Postmortem skin biospies were taken directly below tick feeding sites, fixed in buffered formalin and embedded in paraffin. IHC was done using antibodies against recombinant major surface protein 4 (MSP4) that were indirectly labeled with fluorescein (FA) or peroxidase-antiperoxidase (PAP) and then examined with confocal or light microscopy. Expression of immune response genes, shown previously to be differentially regulated in response to *A. phagocytophilum* infection in sheep, was determined by qRT-PCR in blood and skin biopsies. Variable expression of these genes was observed in tick and non-tick feeding sites of infected and uninfected sheep. Granuloctyes infected with *A. phagocytophilum* were detected in skin biopsies by both IHC methods. Tick feeding appears to attract infected neutrophils and therefore contributes to the exposure and infection of ticks with *A. phagocytophilum* after short feeding periods.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Introduction

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is a gram negative bacteria transmitted by ticks, most notably of the genus *Ixodes*, to a wide range of hosts, including birds, small and large mammals and humans (Goodman 2005; Woldehiwet 2010). This organism is the etiologic agent of a febrile illness of humans (human granulocytic anaplasmosis, HGA), sheep and other ruminants (tick-borne fever, TBF), horses (equine granulocytic anaplasmosis, EGA) and dogs (canine granulocytic anaplasmosis, CGA). While *A. phagocytophilum* is an established pathogen of small ruminants in Europe (Stuen 2007; Stuen et al. 2009; 2013), this pathogen has more recently been shown to be the agent of the emerging tick-borne disease of humans, HGA, in the United States, Europe and Asia (Goodman 2005).

The recognition of the broad distribution of *A. phagocytophilum* and its emergence as a human tick-borne pathogen, particularly in the U.S., have created renewed interest and accelerated research on this organism, particularly on the molecular relationship of the pathogen with its vertebrate and tick hosts (Woldehiwet 2010). Because *A. phagocytophilum* is infective for a wide range of mammals, this pathogen

may emerge in other animal populations in the future, such as food animal species in the U.S., which may impact food animal production and also pose risk of increased exposure of humans.

Research in our laboratory has been directed toward development of a sheep model for studying host-pathogen-vector interactions and the NY-18 human isolate of *A. phagocytophilum*. Sheep were shown to be susceptible to infection with *A. phagocytophilum* and served as a host for infection of *Ixodes scapularis* ticks (Kocan et al. 2012). The research presented in this thesis confirmed our initial findings and extended our understanding of this sheep model by focusing on the tick feeding site and factors which may favor *A. phagocytophilum* infection of the tick vector.

Historical Background and Current Classification

Anaplasma phagocytophilum was first recognized in 1932 as the causative agent of TBF, a disease of sheep first reported in Scotland (MacLeod, 1932). Since that time, this pathogen has been shown to cause disease in sheep and cattle throughout Europe (Hudson 1950). The organism was first named *Rickettsia phagocytophilia* (Foggie 1949), and then was renamed *Cytoecetes phagocytophila* (Foggie 1962) based on morphological similarities to *Cytoecetes microtic* (Tyzzer 1938). Subsequently, this organism was then listed as a separate species, *E. phagocytophila*, and classified in the tribe Ehrlichieae (Ristic and Huxsoll 1984), but this designation was not adopted by researchers in Europe who continued to refer to the organism as *C. phagocytophila* (Woldehiwet and Scott 1993).

Diseases in horses and dogs caused by organisms similar to *E. phagocytophila* were then reported to be emerging in the U.S. The first case of equine granulocytic

ehrlichiosis (EGE) in horses was reported in California in 1969 (Gribble 1969) and was presented as a separate species, *E. equi*. Granulocytic *E. canis,* an emerging disease in dogs, was first recognized in Arkansas in 1971 (Madewell and Gribble 1982). Up to this point, these organisms were thought to be maintained in a transmission cycle between domestic animals and free-living mammalian reservoirs (Ogden et al. 1998 a,b). In the early 1990's, an emerging tick-borne disease causing a febrile illness in humans in the U.S. was shown to be caused by a yet-to-be-named bacteria that parasitized granulocytes in a manner similar to *E. phagocytophila*, *E. equi* and granulocytic *E. canis,* and was named human granulocytic ehrlichiosis (HGE) (Chen et al. 1994).

The disease characteristics of human infections were similar to those described in cases of TBF, EGE and CGE, as well as the unique ability of the organism to invade and survive in host granulocytes, and led to study of the molecular relatedness of organisms in the families, *Rickettsiaceae*, *Bartonellaceae*, and *Anaplasmataceae*. The reclassification of these families that followed was based primarily on the genetic relatedness of the 16S rRNA, groESL, and surface protein genes (Dumler et al. 2001). Phylogenetic analyses resulted in the formation of four distinct genera within the family Anaplasmataceae: (i) *Anaplasma*, with a 96.1% minimum similarity; (ii) *Ehrlichia*, with a 97.7% similarity; (iii) *Wolbachia*, with a 95.6% similarity; and (iv) *Neorickettsia*, with a 94.9% similarity. Most notably, this reclassification resulted in unification of the etiologic agents of EGE, CGE and HGE into one taxon, *A. phagocytophilum*, and the associated diseases (EGE, CGE and HGE) were renamed equine granulocytic anaplasmosis (EGA), canine granulocytic anaplasmosis (CGA) and human granulocytic anaplasmosis (HGA), while *A. phagocytophilum* infection in sheep continued to be

referred to as tick-borne fever TBF. Since this 2001 reclassification many strains of *A. phagocytophilum* have been identified in the U.S., Europe and other areas of the world which differ in host preferences, disease characteristics, molecular composition and other aspects. Each distinct strain is now referred to as a variant of *A. phagocytophilum*.

The bacteria classified within the family Anaplasmatacea are obligate intracellular organisms found exclusively within membrane-bound vacuoles in the host cell cytoplasm. Most organisms within the family Anaplasmataceae multiply within vertebrate and invertebrate hosts. Conversely, organisms classified within the family Rickettsiaceae are obligate intracellular bacteria that are found free within eukaryotic host cell cytoplasm. The reclassification of the genus *Anaplasma* sp. resulted in the placement of *A. phagocytophilum* with organisms that primarily infect ruminants and that are quite host specific (*A. marginale*, *A. centrale* and *A. ovis*). Survival of *Anaplasma* sp. in nature is dependent upon mammalian host reservoirs because transovarial transmission from one generation of ticks to the next via the egg has not been reported, and therefore for tick transmission to occur, ticks must acquire infection as larvae or nymphs and then be transmitted by nymphs or adults.

Epidemiology, geographic distribution and emergence of granulocytic anaplasmosis in the United States and other areas of the world

Anaplasma phagocytophilum, recognized as the most common tick-borne disease of animals in Europe and as an emerging disease of humans in the U.S., has a wide host range and has been reported in mammals and ticks throughout Europe, the U.S. and other areas of the world. The distribution of the organism is dependent upon the presence of the vector, host and reservoir host species (de la Fuente et al. 2005b; Stuen 2007;

Woldehiwet 2010). Likewise, the severity of the disease is dependent upon the strain or variant of the organism and the susceptibility of the host to infection.

Reports of the the number of *A. phagocytophilum* variants have continued to increase worldwide. Recent investigation of sequence variation of the *msp4* gene of *A. phagocytophilum* in 50 samples from the U.S., Germany, Poland, Norway, Italy and Switzerland and four samples from white-tailed deer in the U.S. revealed greater sequence variation in *A. phaogcytophilum* strains as compared with *A. marginale* (de la Fuente et al. 2005b). The results of studies also differentiated strains of *A. phagocytophilum* among ruminants, horses and dogs, and the strains isolated from white-tailed deer were found to be more diverse. These findings were supported by similar studies of sequence analysis of the *msp2* genes. Overall, sequence analysis studies indicate that the human strains differ from ruminant ones and may be maintained in nature within different reservoir hosts (de la Fuente et al. 2005e).

Anaplasma phagocytophilum has been identified in feral ruminant populations in the U.K., and the pathogen has been reported in feral goats and red, fallow and roe deer (Foster and Greig 1969; McDiarmid 1965; Alberdi et al. 2000). The organism has also been isolated from cervids, moose and chamois in Norway, Slovenia, Switzerland and Austria (as reviewed by Woldehiwet 2010). Other species known to become infected with *A. phagocytophilum* include wild rabbits, birds and cats (Bjoersdorff et al. 2001; Daniels et al. 2002; Goehert and Telford 2003; Lappin et al. 2004; de la Fuente et al. 2005c).

Recent research has contributed to an understanding of the epidemiology of *A. phagocytophilum* in southern Europe and Spain. In Sicily, the organism has been detected by PCR analysis of the 16S rRNA gene in a large host range of animals, including cattle, goats, sheep, horses, dogs and mice (de la Fuente et al. 2005d; Torina et al. 2008a; 2010). Further analyses identified five genetically diverse strains that were found in specific geographic locations; two were isolated from hosts exclusively in the western region, one having 98.9% similarity to an isolate from a human case only found in cattle in the eastern region and two genotypes were found in sheep or mice in the eastern region. Such findings provide evidence that domestic animals such as cattle, horses, donkeys, sheep, dogs and cats may serve as reservoir hosts for *A. phagocytophilum.* However, ruminant variants were found to be different from those isolated from humans (Torina et al. 2008b).

Recent investigations have resulted in a better understanding of the epidemiology of *A. phagocytophilum* throughout central, northern and southern Spain. In central Spain, the organism causes disease in humans and is maintained in the environment by cattle, donkeys, deer and birds. In northern Spain, *A. phagocytophilum* infections have been identified in cattle, sheep and humans while wild rabbits, birds and cats have been implicated in the epizootiology of the organism in central and southern Spain (de la Fuente et al. 2005c). PCR analyses and serologic studies identified infection in European roe deer in southern Spain (de la Fuente et al. 2008d). Two distinct *A. phagocytophilum msp4* genotypes were identified in roe deer that had 99.5-99.9% sequence homology to genotypes previously reported from northern Spain, while an 89.9-90.1% sequence homology of genotypes was reported in roe deer from Germany (de la Fuente et al.

2008d). These findings suggest that roe deer serve as an important reservoir host in the natural cycle of *A. phagocytophilum* in Spain.

CGA has also been reported in dogs in Europe, Australia, Canada and all states in the U.S. except Mississippi and Nebraska (as reviewed by Tsachev 2009; Woldehiwet 2010). The organism was first described in dogs in the U.S. prior to its recent discovery in Europe.

Since the first recognition of *A. phagocytophilum* in horses in California, the number of EGA cases has increased throughout the U.S. and other parts of the world, and have been diagnosed in Florida, Colorado, New Jersey and Connecticut (as reviewed by Woldehiwet 2010). *E. equi*, now *A. phagocytophilum*, was identified in the equine population in Scandinavia (Engvall and Egenvall 2002), Switzerland (Pusterla et al. 1998), and the U.K (McNamee et al. 1989; Korbutiak and Schneiders 1994; Shaw et al. 2001).

Prior to the discovery of HGA in the U.S., the disease was thought to be limited to domestic and wild animals. Since the first documented human case of HGA in Wisconsin in the early 1990's, the annual number of human cases in one Wisconsin county alone increased to 58 cases per 100,000 (Dumler et al. 2005). However, many of the newly described strains may be genetically distinct with a limited host range. For example a unique variant (Ap-1) found to be infective for goats and deer was not associated with infection in humans or small mammals (Massung et al. 2006b; Reichard et al. 2009). When ticks that were allowed to feed on deer infected with either the Ap-1 or the human NY-18 isolate, only the ticks that fed on the Ap-1 variant infected deer acquired infection

(Reichard et al. 2009). These studies provided evidence that strains from ruminants share common characteristics that differ from human strains (Reichard et al. 2009; de la Fuente et al. 2005b). While the majority of *A. phagocytophilum* strains are serologically cross reactive, phylogenetic analysis of major surface protein (MSP) sequences, predominately *msp4*, documented strain differences (de la Fuente et al. 2005b).

The majority of HGA cases reported in the U.S. were from areas in which the tick vector, *I. scapularis*, was established, including the northeast and upper mid-west and include Massachusetts, Connecticut, New York, Minnesota and Wisconsin (Rikihisa 2006). A total of 3,637 cases of HGA were reported in the U.S. from 2003 to 2008, with the majority of those cases being identified in 2007 (Thomas et al. 2009). In addition, HGA has been associated with other diseases transmitted by *Ixodes sp.*, including human babesiosis and Lyme disease (Goodman 2005; Nadelman et al. 1997). Serologic evidence suggested that the number of human cases of HGA may be grossly underestimated and that 15%-36% of the population may be infected (Aguero-Rosenfeld et al. 2002; Bakken et al. 1998). A recent report suggested that the incidence of *A. phagocytophilum* increased between 2000 and 2007 from 1.4 to 3.0 cases/million persons/year (Dahlgren et al. 2011).

The first case of HGA in Europe was identified in Slovenia in 1997. Since that time, human cases have been reported in Sicily in 2005 (de la Fuente et al. 2005d). The first Canadian diagnosed with HGA was reported in 2009, which was based on PCR and demonstration of morulae in granulocytes in peripheral blood smears (Parkins et al. 2009). HGA has since been reported in the Netherlands, Spain, Sweden, Norway, Croatia, Poland and Greece (Thomas et al. 2009). Serologic studies suggested the

presence of HGA in adults in Germany, Bulgaria, Spain, Italy, Estonia and Grece, but these cases were most likely to be asymptomatic (Woldehiwet 2010).

Tick vectors, transmission and the tick developmental cycle

Ticks are ectoparasites that infect wildlife, domestic animals and humans and are the most important vector of disease-causing pathogens in some regions of the world (de la Fuente et al. 2008b; Dumler et al. 2001). Ticks from the genera Ixodidae are the primary vectors of *Anaplasma* sp. The tick species responsible for transmission of *A. phagocytophilum* varies depending upon geographic location. In the North America, *A. phagocytophilum* is transmitted by ticks of the *Ixodes persulcatus* complex (Woldehiwet 2010). In the U.S. *I. scapularis* is responsible for transmission of the organism in the Midwest (Pancholi et al. 1995; Goodman 2005), while *I. pacificus* is the tick vector in the western and mountainous U.S. (Richter et al. 1996; Reubel et al. 1998). The tick species most commonly reported to vector *A. phagocytphilum* in Europe is *I. ricinus* (Blanco and Oteo 2002; Strle 2004; as reviewed by Thomas et al. 2009). Other ticks known to transmit *A. phagocytophilum* include *Haemaphysalis punctate, I. persulcatus, I. triangulicep*s and *Rhipicephalus sanguineus* (as reviewed by Steun 2007).

Studies on *I. ricinus* demonstrated that transmission of *A. phagocytophilum* was transstadial (MacLeod and Gordon 1933; MacLeod 1932; 1936). Ticks acquired infection as larvae or nymphs that were allowed to feed on infected hosts, and transmission was effected by subsequent tick stages, nymph or adults. Transovarial transmission via eggs to the next generation has not been reported. Therefore, acquisition of infection by ticks

from infected mammalian hosts during the bloodmeal is required for transmission of *A. phagocytophilum* (Hodzic et al. 1998a).

Advances in molecular techniques have demonstated the potential of tick species other than *Ixodes* to transmit *A. phagocytophilum* and may also contribute to the transmission cycle of the pathogen. A recent study in central Spain evaluated ticks collected from European wild boar (*Sus scrofa*) and Iberian red deer (*Cervus elaphus hispanicus*), and detected *A. phagocytophilum* in *D. marginatus, Rhipicephalus bursa* and *Hyalommam marginatum* (Naranjo et al. 2006; de la Fuente et al. 2004; 2005c,f). The low prevalence of *I.ricinus* in this region of Spain suggested that other tick species may serve as vectors of *A. phagocytophilum,* thus contributing to HGA. While *Dermacentor, Hemaphysalis* and *Rhipicephalus* ticks have been reported as potential vectors of *A. phagocytophilum* (MacLeod 1962; Holden et al. 2003; Alberti et al. 2005a; Cao et al. 2006; Barandika et al. 2008), transmission patterns and target hosts have not been reported for these tick vectors. Interestingly, among the newly identified tick vectors, Baldrige et al. (2009) demonstrated transovarial transmission of *A. phagocytophilum* by *D. albopictus.* This finding is is of interest because transovarial transmission of *A. phagocytophilum* has not been reported previously in *Ixodes sp* ticks. Transovarial transmission would change the transmission pattern and decrease the dependence on mammalian hosts for transmission of *A.phagocytophilum* in nature. These and other findings demonstrated the need for continued research in order to fully understand the significance of newly identified tick vectors and their role in transmission of *A. phagocytophilum.*

Acquisition of *A. phagocytophilum* infection from the mammalian host is dependent upon the parasitemia at the time of tick feeding. Hodzic et al. (1998b) demonstrated that the transmission rate of *A. phagocytohilum* in nymphal *I. scapularis* correlated with the bacteremia level in mouse blood at the time of tick feeding. The number of ticks that became infected with the organism from mice was highest early in the course of disease when parasitemia in circulating granulocytes the greatest and lowest late in the course of disease when parasitemia was minimal (Hodzic et al. 1998 a,b). Interestingly, despite the degree of host parasitemia level, once the ticks became infected *A. phagocytophilum* multiplied and was successfully transmitted (Eriks et al. 1993; Hodzic et al. 1998a). Transmission of *A. phagocytophilum* to a susceptible host occurred within 24 to 48 hours after attachment (Sukumaran et al. 2006). Hodzic et al. (1998b) demonstrated tick infections within 24 hours of attachment with the frequency of tick infection increasing during the period of tick feeding. Ticks that were allowed to feed for 48 hours were able to transmit the infection to mice and those that had fed to repletion had higher infection rates due to both increased acquisiton of organisms in the bloodmeal and increased multiplication of the pathogen within the tick (Hodzic et al. 1998b; Katavolos et al. 1998).

Vertebrate hosts, reservoir hosts and transmission cycle

The transmission of *Anaplasma phagocytophilum* is known to occur between ticks and ruminants but this host range has recently been shown to include a wide range of vertebrate hosts such as rodents, birds, cats, deer, humans, horses, and dogs. Despite this wide host range, only those animals capable of developing persistent infection can serve as competent reservoir hosts (as reviewed by Woldehiwet 2010). The importance of

rodents as potential reservoir hosts is dependent upon the geographic location. In certain regions rodents develop high infection levels and therefore are excellent reservoir hosts for ticks, while in other regions rodents do not develop sufficient parasitemias to effect consistent infection of ticks. The prevalence and severity of disease caused by *A. phagocytophilum* variants are dependent upon the geographic location, presence of reservoir hosts, tick vectors and their ability to effectively transmit the pathogen (Woldehiwet 2010).

The role of mammalian reservoir hosts has been well established and more completely defined in Europe than the United States. The major mammalian reservoirs of *A. phagocytophilum* in Europe include wood mice, yellow-necked mice, voles, roe and red deer (Liz et al. 2000; Petrovec et al. 2002; Silaghi et al. 2008). In the U.S., mammalian reservoirs are those animals susceptible to infection by *I. scapularis,* including white-tailed deer (*Odocoileus virginianus*), raccoons (*Procyon lotor*), white-footed mice (*Peromyscus leucopus*), gray squirrels (*Sciurus carolinensis*) and chipmunks (Levin et al. 2002; Nieto and Foley 2009; Telford et al. 1996; as reviewed by Woldehiwet 2010). Within the U.S., the distribution of the mammalian vectors varies with the geographic location. The white-footed mouse is the major reservoir host in the eastern U.S., while wood rats and the Western gray squirrel are the major reservoir hosts in the western U.S. (Nicholson et al. 1999; Foley et al. 2002; Nieto and Foley 2008; 2009). Recent identification of other closely related species such as Douglas squirrels, flying squirrels and chipmunks, were shown to be susceptible to *A. phagocytophilum* and also may harbor ticks that are capable of transmitting the organism to other animals and humans (Foley et al. 2007; 2008 a,b; Nieto and Foley 2009).

The role of birds as potential reservoir hosts has not been fully investigated. However, at least two species of birds within the U.S. may be hosts for variants of *A. phagocytophilum* and may also serve as a source of infection for larval ticks (Daniels et al. 2002). In Sweden, *A. phagocytophilum* infected *I.ricinus* nymphs were collected from migrating birds (Bjoersdorff et al. 2001). In Spain, the role of birds as a potential source of *A. phagocytophilum* was evaluated by testing blackbirds and *Turdus spp.* using an *A. phagocytophilum* specific PCR assay (de la Fuente et al. 2005e). In another study conducted on a large hunting estate in central Spain, blackbirds were found to have the highest prevalence of *A. phagocytophilum* by PCR (de la Fuente et al. 2005c). These results suggest that birds could contribute to the epidemiology of *A.phagocyptophilum* by serving as both reservoir hosts and also by spreading infected ticks during migrations.

Transplacental transmission

While *A. phagocytophilum* is an economically important pathogen of sheep in Europe (Stuen, 2007; Stuen et al, 2009), the pathogen has more recently been shown to cause the emerging tick-borne disease of humans, HGA, in the United States, Europe and Asia (Goodman, 2005). For the type species, *A. marginale*, three means of transmission occur (tick-borne, mechanical transmission by blood contaminated fomites and mouthparts of biting arthropods and transplacental transmission), but the latter two (mechanical and transplacental) have not been considered as a means of *A. phagocytophilum* transmission. However, transplacental transmission of *A. phagocytophilum* was reported previously in an experimentally infected cow (Pusterla et al, 1997) and a naturally-infected human (Dhand et al, 2007; as reviewed by Horowitz et al, 1998). Despite such findings, documented cases of transplacental transmission have

not been reported in sheep. Sheep are capable of becoming persistently infected with the organism. Therefore, transplacental transmission may be a cause of TBF in lambs that have not been exposed to the tick reservoir (Thomas et al. 2012) and warrants further investigation.

Pathogenesis and clinical presentation

Anaplasma phagocytophilum multiples and survives within mammalian cells of the granulocytic lineage. This obligatory intracellular pathogen multiplies in parasitophorous vacuoles called a morula in the host cell cytoplasm. Morulae of *A. phagocytophilum* are approximately 1.5 µm to 2.5 µm in diameter but have reported to be as large as 6 µm (Popov et al. 1998). Infection of mammalian host cells is dependent upon *A. phagocytophilum* recognition of the host cell receptor. However, the exact mechanisms by which the organism infects the host dermal tissue at the tick feeding site and their host cells resulting in clinical signs of disease are not well understood (Goodman 2005).

The cell types targeted for infection during the period of bacteremia are eosinophils, neutrophils and macrophages. Neutrophils serve as the first line of defense against invading organisms and typically have a life span of 6-12 hours (Savill 1989; Akgul 2001). Due to their short life span, as well as their ability to phagocytize infecting bacterial organisms, neutrophils are not considered a hospitable environment for intracellular bacteria. However, *A.phagocytophilum* has evolved mechanisms for evading the hosts defenses while, at the same time, promoting attraction of neutrophils to the tick feeding site (Granquist et al. 2010). An additional function of neutrophils is their ability

to undergo apoptosis in response to bacterial killing. *A. phagocytophilum* infection in isolated human peripheral blood neutrophils was shown to inhibit spontaneous and induced apoptosis for up to 48 h. This effect was also seen in morphologic evaluation of neutrophils in peripheral blood leukocyte cultures for up to 96 h (Niu et al. 2010). The ability to manipulate neutrophil function is critical for propagation and replication of the organism within the host. After infection of cells and establishment in the parasitophorous vacuole, *A. phagocytophilum* undergoes multiplication until the cell lyses and organisms are released to subsequently infect other cells.

The early development of *A.phagocytophilum* within the mammalian host has not been clearly described. A prepatent period of 4-7 days occurs between transmission from ticks to development of rickettsemia. However, the exact location of *A. phagocytophilum* of this initial development within the host presently is not known. In experimental studies, susceptible animals inoculated with infected cells did not develop detectable rickettsemias until 72-96 hours post inoculation (Woldehiwet 2010). This data suggested that prior to visualization of morula in stained blood smears the organism may remain undetectable because of initial replication within a different host cell. Recent studies suggested that *A. phagocytophilum* can be isolated from lung and spleen tissues prior to detection of the organism in the peripheral blood (Snodgrass 1974; Woldehiwet 2010). Other evidence suggests that *A.phagocytophilum* is more likely to infect myeloid precursors as compared with mature neutrophils (Walker and Dumler 1996; Woldehiwet 2010). Despite this evidence, early studies in sheep did not support infection of immature neutrophils with *A. phagocytophilum* (Woldehiwet and Scott 1982; Woldehiwet 2010). In one study, sheep infected with *A. phagocytophilum* were treated with dexamethasone

during peak rickettsemia, and, while there was a dramatic increase in the number of circulating granulocytes, the percentage of infected neutrophils actually decreased. Such results suggested that immature neutrophils mobilized from bone marrow were not infected prior to peripheral blood neutrophils (Woldehiwet 2010).

Infection of host granulocytes with *A. phagocytophilum* may result in high parasitemias, but the severity of clinical signs and duration of disease is dependent upon the strain of *A.phagocytophilum* and the susceptibility of the host (Foggie 1951; Tuomi 1967a,b; Woldehiwet and Scott 1982;1993; Woldehiwet 2010). In domestic ruminants and horses, the first clinical sign of disease is a persistent fever that lasts for a minimum of 7 days (Tuomi 1967a,b,; Gribble 1969; Woldehiwet 1987a; Woldehiwet 2010). Fever detected in sheep recently after being moved to tick-infested pastures is considered an indicator of TBF. Other clinical signs associated with *A. phagocytophilum* include pyaemia in lambs, respiratory disease in cattle, decreased milk production and secondary infections (Woldehiwet 2006). Severe leukopenia and prolonged neutropenia are additional indicators of TBF. In addition, ewes in late gestation that are moved to tick-infested pastures are at increased risk of abortion secondary to infection with *A. phagocytophilum* (Woldehwiet 2006). The most common clinical signs of canine and equine granulocytic anaplasmosis include fever, depression, anorexia, leukopenia and thrombocytopenia (Dumler et al. 2005).

The clinical presentation of HGA in humans is characterized by fever, chills, headache and myalgia along with a history of recent tick bites 1-2 weeks prior to the development of clinical disease (Bakken and Dumler 2006). The spectrum of clinical signs associated with HGA ranges from asymptomatic to a severe acute febrile illness

leading to death in some cases (Goodman 2005). However, as suggested by Dumler et al. (2005), human infections may not always be apparent and 15%-36% of the population in tick endemic areas could be infected without apparent clinical signs.

One major effect of *A. phagocytophilum* in the pathogenesis of granulocytic anaplasmosis is modification of host cell neutrophil function with inhibition of cellular apoptosis. Interestingly, when *A. phagocytophilum* was propagated in the human promyelocytic cell line, HL-60 cells, apoptosis of the infected cells did not appear to be inhibited at high infection levels. In this instance, infection with *A. phagtocytophilum* resulted in the majority of host cells dying or undergoing degenerating (de la Fuente et al. 2005a). Such findings suggest that the anti-apoptic effect of *A.phagocytophilum* is neutrophil-specific and not a global phenomenon (de la Fuente et al. 2005a).

Clinical and Laboratory Diagnosis

Historically, clinical diagnosis of *A. phagocytophilum* has been based on the presence of an acute febrile illness with histologic evidence of morula within granulocytes on a blood smear during rickettsemia. Visualization of morula in human granulocytes is frequently observed but is not a consistent finding among other hosts. Therefore, serology and polymerase chain reaction (PCR) amplification of *A. phagocytophilum* DNA from acute-phase blood are required to definitively diagnose infection. *A. phagocytophilum* infection can also be confirmed by isolation of the human promyleocytic cell line, HL-60, inoculated with with acute-phase blood. However, all tests utilizing blood samples must be performed prior to the initiation of antimicrobial therapy which would cause a rapid decrease the rickettsemia (Bakken and Dumler 2006).

The importance of rapid detection of infection is to provide rapid treatment of the condition (Chandrashekar et al. 2010).

In 2010, Chandrashekar et al. (2010) evaluated the efficacy of a commercially available cELISA for identification of *A. phagocytophilum.* The cELISA was based on the immunodominant P44 protein. Dogs that had been experimentally infected with *A. phagocytophilum* were seropositive within 8 days post inoculation despite the lack of a fever or the presence of morulae within granulocytes on a peripheral blood smear. A positive ELISA indicated exposure to the pathogen but PCR was needed to definitively confirm active infection. Interestingly, the dogs in this study also had antibodies to *A. platys* which cross-reacted with the *A. phagocytophilum* ELISA which provided evidence that the ELISA is capable of detecting *Anaplasma* sp. but not differentiate between species. The serologic cross-reactivity was shown to be the result of conserved surface proteins, most notably major surface protein 5 (MSP5). An ELISA developed using a monoclonal antibody against MSP5 is a component of the *A. marginale* ELISA if the approved test for detection of bovine anaplasmosis in the U.S. and Canada (Dreher et al. 2005; Strik et al. 2007), and this cELISA was also shown to be cross-reactive with *A. phagocytophilum* antibodies.

Indirect fluoresecent antibody (IFA) tests have also been developed for serologic diagnosis of *A. phagocytophilum* (Chandrashekar et al. 2010). Using IFA dogs were found to seroconvert as early as 2-4 days after first appearance of morulae within the blood.

Propagation in cell culture

A. phagocytophilum has been experimentally cultured in endothelial cell lines from several species, including human, monkey and bovine cell lines (Munderloh 2004). The human promyelocytic cell line allowed for direct isolation of the organism from humans and mice (Blas-Machado et al., 2007, Goodman et al, 2005). Two tick strains isolated from embryos of *I. scapularis* have also been used to propagate human and other strains of *A. phagocytophilum* (Woldehiwet and Horrocks 2005; Munderloh et al. 1996 a,b; 1999; Massung et al. 2006a; Reichard 2009). Cell culture has provided an in vitro system for characterization of pathogen adhesion and gene expression (Goodman et al. 1999; Jauron et al. 2001; Woldehiwet and Horrocks, 2005), as well as human and tick cell responses to infection (de la Fuente et al. 2010; Villar et al. 2010; Zivkovic et al. 2009; 2010; de la Fuente et al. 2007b).

Genetic variants of *A.phagocytophilum*

Many *A. phagocytophilum* variants have been identified by sequence analysis of key genes in a variety of hosts. Comparison of TBF and HGA variants was done by analysis of the 16S rRNA gene, and TBF variants were found to differ in three positions as compared with HGA variants (Chen et al. 1994). Despite the presence of different variants, identical 16S rRNA sequences were shown for variants that were isolated from HGA patients in the U.S. (Belongia et al. 1997; Massung et al. 2002; 2003; 2005). Sequence analysis of the *msp4* gene of *A. phagocytophilum* has also been used to differentiate variants from dogs, horses and humans (de la Fuente et al. 2005b). Genetic variation of *A. phaocygophilum* was demonstrated among sheep from the same flock

(Stuen et al. 2002). The majority of these variants are serologically cross-reactive because of outer membrane proteins which are highly conserved (Dumler et al. 1995; Zhi et al. 1997; 1998).

The pathogenicity *A. phagocytophilum* variants ranged from nondetectable to causing notable clinical signs (Gabriel et al 2009; Madigan et al. 1995; Morissette et al. 2009; Foley et al. 2002; 2007; 2008 a,b; Nieto and Foley 2008; 2009; Goodman 2005). While mechanisms responsible for these variations in pathogenicity have not been fully elucidated (Rikihisa 2011), the differences are important to define in order to better understand the epidemiology and ecology of *A. phagocytophilum* in a wide range of hosts and geographic locations.

Genomics

The genome of the human HZ isolate of *A. phagocytophilum* was sequenced and determined to be 1.47 Mb (Rikihisa et al. 1997), which is much smaller than the genome of *Escherichia coli.* Further characterization of the genome revealed several open reading frames (ORFs: 1,369), but no plasmids, intact prophages or transposable elements were identified (Rikihisa 2011). The genes necessary for synthesis of lipopolysaccharide and peptidoglycan were also found to be absent from the genome (Lin and Rikihisa 2003; Dunning Hotopp et al. 2006).

The immunodominant major surface proteins of *A. phagocytophilum* were found to be contained within the *p44/msp2* multi-gene family (Wuryu et al. 2009), and the Omp-1/P44/Msp2 superfamily has been the most intensively study outer surface protein. The genome of *A. phagocytophilum* has multiple repeats totallying over 100 p44/*msp2*

genes and genes with tandem repeats (Dunning Hotopp et al. 2006; Storey et al. 1998). The transcription of p44 genes contributes to the antigenic diversity of different variants of *A. phagocytophilum* and is important for the pathogenicity of the organism. The variation in these genes and related surface proteins may reflect differences among strains in geographic regions and host specificities (Lin et al. 2004).

The ability of *A. phagocytophilum* to alter the expression of different genes when exposed to different environments may contribute to the development of novel gene function or pseudogenes (Lin et al. 2004). The *A. phagocytophilum* genome has 121 genes within this superfamily: one *msp2,* two *msp2* homologs, one *msp4,* 113 *p44,* and three *omp-1* genes. *A. phagocytophilum* genes are differentially expressed in HL-60 and ISE3 cultured cells, suggesting that the host cell environment is capable of regulating gene transcription (Wang et al. 2007; Nelson et al. 2008, Galindo et al. 2008; Zivkovic et al. 2009).

Tick vector/host interactions at the tick feeding site

Ticks serve as the most important cause of vector borne disease in animals (Balashov, 1972) and have developed ways to modulate the host immune response resulting in immunosuppression of the host which promotes survival of both the tick and the pathogen.The long duration of tick attachment and feeding impacts the host immune system at the feeding site, including increased vascular permeability, activation of the coagulation cascade, increased infiltration of inflammatory cells and stimulation of the innate and acquired immune systems (Francischetti et al., 2010). In response, the host has developed methods of resistance to tick infestation which result in reduced engorgement

weight, increased duration of feeding, decreased number and viability of ova and, in some cases, tick death (Wikel, 1999). A wide range of modifications by the host resulting from pathogen infection may further result in alterations of the acquired immune response including activation and mobilization of eosinophils, basophils, antigen presenting cells, complement, B and T-lymphocytes, cytokines, and other circulating granulocytes to the tick bite site (as reviewed by Wikel, 1999). Both the pathogen and tick vector are capable of modifying the host's innate and acquired immune response which is critical for the propagation and survival of the pathogen and survival and feeding of the tick, processes that are perfectly coordinated (Zeinder et al., 1996). *Ixodes scapularis* mediated immunosuppresion involves inhibition of complement components C3b and C5b, salivary inhibition of C3 hydrolysis leading to decreased release and activation of mast cell mediators, as well as inhibition of bradykinin necessary for pain and increased vascular permeability at the tick bite site (Ribeiro, 1987; Ribeiro and Spielman, 1986; Ribeiro et al., 1985; Ribeiro and Mather 1998).

Sheep as model host for *A. phagocytophilum*/tick interactions.

TBF is an economically important disease of sheep in Europe and therefore has been studied extensively, and natural *A. phagocytophilum* infections in sheep have been well documented. Experimentally, sheep have been shown to be a good host for raising and feeding ticks. Research in our laboratory was initiated to develop sheep model for studying tick/host/pathogen interactions of *I. scapularis* and the human NY-18 isolate of *A. phagocytophilum* (Kocan et al., 2012). For this model sheep were inoculated with the NY-18 isolate of *A.phagocytophilum* propagated in the HL-60 cell line. Six week after *A. phagocytophilum* infection was detected by PCR, sheep were infested with adult *I.*

scapularis. The majority of the ticks that were allowed to feed on experimentally-infected sheep were found by PCR to be infected with *A. phagocytophilum* after feeding periods as short as two days. However, none of the infected sheep developed clinical signs of disease and morulae were not observed in stained blood smears (Kocan et al., 2012). While the lack of clinical signs was in contrast to sheep that have been experimentally or naturally acquired TBF, this study demonstrated that sheep can be used as a host for infection of *I. scapularis* with this human isolate. The fact that clinical signs were not observed in sheep and infected granulocytes were not seen in stained blood smears, raised the question of the host cell that was the source of infection for ticks. Therefore, the research focused on study of the tick feeding sites in order to better understand tick/host/pathogen interactions and the source of *A. phagocytophilium* infection for ticks.

Summary

While *A. phagocytophilum* has been a pathogen of veterinary importance in Europe for over 70 years known as the cause of TBF sheep, the recent emergence of HGA and increased incidence of this disease in the United States, Asia and Europe, has generated global interest conducting research on this pathogen. The transmission cycle has historically been thought to be primarily between ticks and ruminants. However, the host range has broadened to include a wide variety of *A. phagocytophilum* variants and hosts, including rodents, birds, cats, deer, horses, cattle, dogs, sheep and humans. Recent research provided evidence that the pathogenicity of variants is influenced by interactions between the *A. phagocytophtilum* strain, vertebrate host(s), the tick vector and the geographic location. Identification of variants has been done by PCR and sequence analysis because serologic cross-reactions result from the presence of highly conserved

surface proteins. *A. phagocytophilum* has a wide host range and the overall risk of infection with *A.phagocytophilum* and the spread of granulocytic anaplasmosis will likely continue to increase because of expanding populations and the changing distribution of *Ixodes* sp, a concern for both human and animal health in the U.S.

Granulocytes are the target cell for *A.phagocytophilum* in the vertebrate host. Within the cell, the organism resides within a parasitophorous vacuole or morula which is identified on a peripheral blood smear. The molecular mechanisms associated with early infection have not been well established. Therefore, during early infection, the organism remains undetectable and may replicate within another host cell that has not yet been identified. Infection of host cells with *A. phagocytophilum* has been found to result in transcriptional changes and altered cell functions that may contribute to the pathogenesis of the disease.

The tick is a required vector in the life cycle of *A. phagocytophilum*. The exact developmental cycle of the organism within the tick is not completely defined. Recent research has contributed to further development of the exact interaction between the tick cell and the pathogen. However, continued studies are needed to more completely understand this interaction and the impact of infection on vector competency and pathogen transmission.

Recent research has focused largely on the molecular interaction of *A. phagocytophilum* and the host cells. The pathogen has evolved unique methods to exploit and invade host cells. Development of a sheep model of the human NY-18 isolate of *A. phagocytophilum* provides the opportunity to study interaction of the tick/pathogen/host

interactions in order to further define the invasive nature of the organism and the mechaisms by which ticks acquire infection. These studies are especially important in this sheep model where infected granulocytes were not seen in stained blood smears, but in which ticks readily acquired *A. phagocytophilum* infection after a short feeding period. This finding prompted our interest in characterizing the tick feeding site to better understand the relationship between the host, tick vector and pathogen. The current research was, therefore, focused on the tick feeding site in order to define the relationship between the tick feeding and the host response and to determine the cellular source of *A. phagocytophilum* infection for ticks.

RESEARCH PROBLEM

HGA, caused by *A. phagocytophilum,* is an emerging tick-borne disease in the U.S. The increasing incidence of HGA has generated renewed interest in research on this pathogen in order to more clearly define the relationship between the host, the vector and the pathogen in order to improve disease prevention and treatment. In natural infections, *A. phagocytophilum* is transmitted by *Ixodes* ticks and infects host cells of the granulocytic lineage. Clinically, ruminants exhibit an acute febrile illness and infection is confirmed during febrile episodes by detection of morulae within granulocytes in stained blood smears. We recently established a sheep model for studying the tick/pathogen/host interactions of a U.S human isolate of *A. phagocytophilum* obtained from a clinical case of HGA. In this sheep model while sheep were experimentally infected by inoculation with the NY-18 isolate that was propagated in HL-60 cells, they did not develop clinical disease and the histopathological response was mild. Surprisingly, these sheep served as an efficient source of infection for ticks. The lack of circulating granuocytes infected with *A. phagocytophilum* suggested that these cells are attracted to and sequestered at the tick feeding site. The focus of this research was therefore to further characterize tick feeding sites in order to describe the environment in which ticks readily acquired *A. phagocytophilum* infection and led to formulation of the hypothesis for this research that the host-pathogen interactions at the tick feeding site modulate infection of ticks with the human NY-18 isolate of *A. phagocytophilum*.

The specific objectives are as follows:

1. To confirm sheep as a model host for infection of ticks with the human NY-18 isolate of *A. phagocytophilum.*

2. To characterize the histopathology of the tick feeding site from sheep experimentally infected with the human NY-18 isolate of *A. phaogcytophilum.*

3. To characterize *A. phagocytophilum* infections at the tick feeding site.

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CHAPTER II

DEMONSTRATION OF TRANSPLACENTAL TRANSMISSION OF A HUMAN ISOLATE OF *ANAPLASMA PHAGOCYTOPHILUM* IN AN EXPERIMENTALLY INFECTED SHEEP

SUMMARY

Anaplasma phagocytophilum, first identified as a pathogen of sheep in Europe, has more recently been recognized as an emerging tick-borne pathogen of humans in the U.S. and Europe. Transmission of *A. phagocytophilum* is reported to be by ticks, primarily of the genus *Ixodes*. While mechanical and transplacental transmission of the type genus, *A. marginale*, occur in addition to tick transmission, these modes of transmission have not been considered for *A. phagocytophilum*. Recently, we developed a sheep model for studying host/tick/pathogen interactions of the human NY-18 *A. phagocytophilum* isolate. While sheep were susceptible to infection with this human isolate in our studies and served as a source of infection for *I. scapularis* ticks, they did not display clinical signs of disease, and the pathogen was not apparent in stained blood smears. In the course of these *A. phagocytophilum*/sheep experiments, one sheep unexpectedly gave birth to a lamb 5 weeks after being experimentally infected by inoculation with the pathogen. The lamb was depressed and was subsequently euthanized 18 hrs after birth. Tissues were collected

at necropsy for microscopic examination and PCR in order to confirm *A. phagocytophilum* infection. At necropsy the stomach contained colostrum, the spleen was moderately enlarged and thickened with conspicuous lymphoid follicles and mesenteric lymph nodes were mildly enlarged and contained moderate infiltrates of eosinophils and neutrophils. Blood, spleen, heart, skin, cervical and mesenteric lymph nodes tested positive for *A. phagocytophilum* by PCR, and sequence analysis confirmed infection of the lamb with the NY-18 isolate. Transplacental transmission should therefore be considered as a means of *A. phagocytophilum* transmission and may likely contribute to the epidemiology of tick-borne fever in sheep and other mammals, including humans.

Key Words: *Anaplasma phagocytophilum*, transplacental transmission, human isolate, sheep, tick-borne

Introduction

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is a tick-borne pathogen infective for a wide range of hosts, including birds, small and large mammals and humans (Goodman, 2005; Woldehiwet, 2010). This organism is the etiologic agent of a febrile illness of humans (human granulocytic anaplasmosis, HGA), sheep and other ruminants (tick-borne fever, TBF), horses (equine granulocytic anaplasmosis, EGA) and dogs (canine granulocytic anaplasmosis, CGA). While *A. phagocytophilum* is an economically important pathogen of sheep in Europe (Stuen, 2007; Stuen et al, 2009), the pathogen has more recently been shown to cause the emerging tick-borne disease of humans, HGA, in the United States, Europe and Asia (Goodman, 2005). For the type species, *A. marginale*, three means of transmission occur (tick-borne, mechanical transmission by blood contaminated fomites and mouthparts of biting arthropods and transplacental transmission), but the latter two (mechanical and transplacental) have not been considered as a means of *A. phagocytophilum* transmission. However, transplacental transmission of *A. phagocytophilum* was reported previously in an experimentally infected cow (Pusterla et al, 1997) and a naturally-infected human (Dhand et al, 2007; as reviewed by Horowitz et al, 1998).

We recently developed a sheep model for a human isolate, the NY-18 isolate, of *A. phagocytophilum* in order to study molecular host/tick/pathogen interactions (Kocan et al, 2012). While sheep were susceptible to infection with this human isolate and served as a source of infection for *I. scapularis* ticks, they did not display clinical signs of disease and the pathogen was not conclusively demonstrated in stained blood smears. However, the sheep served as a good host for infection of ticks, with the infection rates ranging

from 80-100% after a feeding period as short as 2 days. In the course of recent experiments, one sheep unexpectedly gave birth to a lamb 5 weeks after being experimentally infected by inoculation with *A. phagocytophilum* infected cell cultures, near the beginning of the 3rd trimester of gestation. Herein, we describe transplacental transmission of *A. phagocytophilum* infection to the lamb.

Materials and Methods

Experimental design overview

Sheep No. 44, one of several sheep in a larger study, was experimentally infected with *A. phagocytophilum* by intravenous inoculation (iv) of HL-60 cell cultures infected with the human NY-18 isolate of *A. phagocytophilum* (Asanovich et al, 1997; de la Fuente et al, 2006). The pregnancy status of Sheep 44 had not been determined prior to the onset of the experiment. The sheep was monitored for infection by daily recording of clinical signs, PCR of blood samples, and by examination of stained blood films. A lamb, born unexpectedly to Sheep 44, five weeks after being inoculated with *A. phagocytophilum*, was tested for infection.

Anaplasma phagocytophilum isolate, propagation in HL-60 cells and infection of sheep

The human NY18 isolate of *A. phagocytophilum* (Asanovich et al, 1997; de la Fuente et al, 2006) was propagated in cultures of the human undifferentiated promyelocytic cell line, HL-60. Infected and uninfected cell cultures were maintained at 37°C in RPMI medium as reported previously (de la Fuente et al, 2005a). For inoculation of each sheep, two T-25 flasks of *A. phagocytophilum*-infected HL-60 cells were used (45% infection, as determined by detection of intracellular morulae in stained cytospin cell smears;

Hema-3 Stain, Fisher Scientific, Middletown, VA, USA). The cultures were centrifuged and resuspended with serum free RPMI 1640 medium with a final iv dose of 1 X 10^7 cells in 2 ml of cell culture medium.

Sheep 44, one of a group of sheep used for a tick infection experiment, was purchased at a local livestock auction for use in this study. The sheep was first determined to be negative for *A. phagocytophilum* by PCR analysis of blood samples and subsequently inoculated with HL-60 cell cultures infected with *A. phagocytophilum* and used for tick feeding experiments (Kocan et al, 2012). Blood and serum samples were collected 2 times per week, and stained blood films were prepared and examined on blood collection days for the presence of characteristic *A. phagocytophilum* morulae in granulocytes.

Necropsy, collection of tissues and PCR studies

A blood sample was collected from the lamb prior to euthanasia and samples of spleen, liver, heart, skin, lung, cervical and mesenteric lymph nodes were collected at necropsy. DNA was extracted from blood, spleen, liver, heart, skin, lung, cervical and mesenteric lymph nodes, using TriReagent (Sigma, St. Louis, MO, USA) following manufacturer's recommendations. *A. phagocytophilum* infection levels were characterized by *msp4* PCR using the Applied Biosystem 2720 thermocycler as described previously (de la Fuente et al, 2006), using oligonucleotide primers MSP4-L (5' - CCTTGGCTGCAGCACCACCTG-3') and MSP4-R (5'- TGCTGTGGGTCGTGACGCG-3') (Busby et al, 2011). PCR reaction products were analyzed by agarose gel electrophoresis to determine positive samples. To corroborate the identity of the *A. phagocytophilum* isolate, *msp4* amplicons were resin purified (Wizard; Promega) and cloned into pGEM-T vector (Promega) for sequencing (Core Sequencing

Facility, Department of Biochemistry and Molecular Biology, Oklahoma State University). Samples of the same tissues were also fixed in buffered formalin for microscopy. Quantitative PCR was also performed to test for difference in infection levels among the tissues as previously described by Zivkovic et al. 2009, but normalizing against *Ovis aries* Aldolase B (gene bank accession number NM_001009809) using primers Oa-ALDOBF (5' -CCCATCTTGCTATCCAGGAA -3') and Oa-ALDOBR (5'-TACAGCAGCCAGGACCTTCT -3').

Results and Discussion

The lamb born to Sheep 44 was infected with *A. phagocytophilum* at birth, the infection being acquired presumably by transplacental transmission. In our previous studies, the prepatent period observed in experimentally infected adult sheep varied from 10-21 days, suggesting it was unlikely that the newly borne lamb could have become infected from ingesting colostrum immediately after birth. Although clotted colostrum was found in the lamb's stomach at necropsy, the lamb was clearly depressed and feeding had not been observed. Furthermore, tissues (spleen, liver, heart, skin, lung, cervical and mesenteric lymph node), collected at the necropsy approximately 18 hours after birth, were all PCR positive by *A. phagocytophilum msp4* gene PCR with the exception of lung tissue samples which were negative (Fig. 1). Infection levels in these PCR positive tissue samples, as determined by quantitative PCR, were not significantly different.

Lesions seen at necropsy were similar to those described in our previous studies (Kocan et al, 2012), and were mild and restricted to the lymphoid system. The spleen was moderately enlarged and thickened and mesenteric lymph nodes were mildly enlarged.

Microscopic examination of spleen showed a mildly increased number of neutrophils in the red pulp. Lymph nodes displayed moderate sinusoidal eosinophilia and neutrophilia. While transplacental transmission of *A. phagocytophilum* has been reported in one naturally infected human (Horowitz et al, 1998; Dhand et al, 2007) and one experimentally infected cow (Pusterla et al, 1997), this means of transmission has not been reported or studied in sheep. However, sheep have been clearly shown to become persistently infected with *A. phagocytophilum,* thus increasing the likelihood of transplacental transmission, and this means of transmission may be the cause of infections in lambs in the absence of natural tick challenge-exposure (Thomas et al, 2012). While the seroprevalance of lambs was found to negatively correlate with the mean meters above sea level (masl), infected lambs were still present. In addition, infected lambs were observed on farms in which lambs were treated with acaricides for tick control, as well as in tick-free pastures or high altitude pasturing where ticks would not be present (Hardeng et al, 1992; Stuen and Bergstrom, 2001; Grøva et al, 2011; Stuen et al, 2012).

The present study further documents transplacental transmission of *A. phagocytophilum*, but additional studies are needed to assess the impact of this mode of *A. phagocytophilum* transmission in sheep and other hosts. Transplacental transmission may prove to be an important means of spread of *A. phagocytophilum* in many host species and thus contribute to the epidemiology of this pathogen, especially in the absence of the tick vector.

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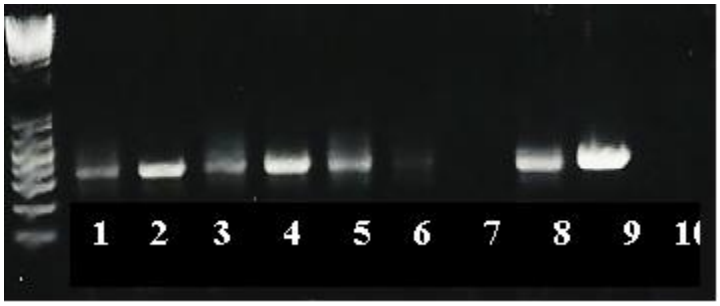
Figure 1. *A. phagocytophilum msp4* PCR products of tissues collected from the lamb born to Sheep 44. Lane 1, blood, Lane 2, mesenteric lymph node; Lane 3, blood; Lane 4, spleen; Lane 5, heart; Lane 6, cervical lymph node; Lane 7, lung (negative), Lane 8, skin; Lane 9; *A. phagocytophilum* DNA (positive control); Lane 10, negative control.



CHAPTER III

STUDIES OF *ANAPLASMA PHAGOCYTOPHILUM* IN SHEEP EXPERIMENTALLY INFECTED WITH THE HUMAN NY-18 ISOLATE: CHARACTERIZATION OF THE TICK FEEDING LESIONS

Abstract

Anaplasma phagocytophilum, transmitted by ticks of the genus *Ixodes*, was first described in Scotland as the agent of tick-borne fever in sheep and more recently as the cause of human granulocytic anaplasmosis in the U.S. and Europe. We previously reported sheep as an experimental host for the human NY-18 isolate of *A. phagocytophilum.* While clinical signs were not observed and morulae in granulocytes were not seen in stained blood smears, these sheep served as a good host for infection of ticks. In this research we characterized tick feeding sites to better understand tick/host/pathogen interactions. *Ixodes scapularis* adults were allowed to feed for 2 and 4 days on experimentally infected sheep, after which biopsies were taken beneath tick feeding sites for histopathology, PCR and immunohistochemistry (IHC) studies. In addition, the expression of selected immune response genes was studied in blood and feeding site biopsies. While necrosis was too advanced in 4-day biopsies for accurate cell counts, higher numbers of eosinophils and neutrophils were found in 2-day biopsies from infected sheep as compared with the

uninfected controls. An unexpected result was the documentation of higher dermal inflammation in infected sheep at sites without ticks. *A. phagocytophilum* infected granulocytes were localized by immunohistochemistry (IHC) in skin biopsies using rabbit antibodies against the recombinant *A. phagocytophilum* major surface protein 4 as the primary antibody for indirect peroxidase-antiperoxidase and fluorescent antibody IHC. These infected cells are likely to be the source of infection for ticks. Sheep therefore served as good hosts for studying host/pathogen/tick interactions of this human strain of *A. phagocytophilum*, and provided a means of producing infected ticks for future studies on tick/pathogen developmental and transmission cycles.

Key words: *Anaplasma phagocytophilum, Ixodes scapularis,* tick bite site, NY-18 human strain, iummunocytochemistry.

Introduction

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is a pathogen transmitted primarily by ticks of the genus *Ixodes* to a wide range of hosts. Although *A. phagocytophilum* transmission was thought previously to be primarily between ticks and ruminants (Woldehiwet, 2010), the host range is now known to include rodents, hedgehogs, birds, cats, deer, horses, cattle, dogs, sheep, and humans (Stuen et al. 2013; Dumitrache et al. 2013). After the reclassification of the Family Anaplasmataceae by Dumler et al. (2001), *A. phagocytophilum* has been recognized as the etiologic agent of several diseases including human granulocytic anaplasmosis (HGA), tick-borne fever (TBF) in sheep and other ruminants, equine granulocytic anaplasmosis (EGA) in horses and canine granulocytic anaplasmosis (CGA) in dogs (Dumler et al. 2001; Goodman, 2005; Woldehiwet, 2010; Stuen et al. 2013). However, while several organisms were

combined as *A. phagocytophilum*, genetic analyses have demonstrated notable strain variation among *A. phagocytophilum* variants and differences were found between ruminants, horse, dogs and humans. These and other studies have suggested that strains in ruminants may share common characteristics which differ from human strains (de la Fuente et al. 2005b; Torina et al. 2008; Reichard et al. 2009; Rar and Golovljova 2011).

In Europe *A. phagocytophilum* is an economically important pathogen of sheep and infection causes septicemia resulting from secondary infection, abortion, as well as lameness in lambs (Stuen, 2007; Stuen et al. 2009; Grøva et al. 2011). During the rickettsemia in sheep, neutrophils were found to be the main host cell for *A. phagocytophilum*, but eosinophils and monocytes were also present, and notable parasitemias of circulating granulocytes (≤ 90%) were determined by examination of stained blood smears (Foggie, 1951; Tuomi, 1967 a,b; Woldehiwet and Scott, 1982; 1993; Woldehiwet 1987; Woldehiwet, 2010). Recently, neutrophils infected with *A. phagocytophilum* were also demonstrated with IHC in skin biopsies taken at the tick feeding lesions from the naturally infected lambs (Granquist et al. 2010).

The emergence of *A. phagocytophilum* as a human pathogen in the U.S., Europe and Asia (Goodman, 2005) has drawn interest in characterizing human isolates. Since studies of human isolates require an animal model, we initiated studies to determine whether sheep would serve as an experimental host for defining host/pathogen/tick interactions. Our initial studies confirmed that sheep were susceptible to infection and served as a source for infection of *I. scapularis* ticks (Kocan et al. 2012; Reppert et al. 2012). In contrast to infections in naturally and experimentally infected sheep in Europe (Stuen, 2007; Stuen et al. 2009), infection of sheep with this human strain did not result in clinical signs and

infected granulocytes were not seen in stained blood smears. However, *I. scapularis* females that were allowed to feed on these sheep acquired infection after short feeding periods (2-4 days). This finding prompted our interest in characterizing the tick feeding site to better understand the relationship between the host, tick vector and pathogen. In this research we characterized tick feeding sites on sheep infected with the NY-18 isolate of *A. phagocytophilum* and provided evidence for the source of *A. phagocytophilum* infection for ticks that fed on the experimentally infected sheep.

Materials and Methods

Experimental Design Overview

The experimental design overview is shown in Figure 1. Three sheep (44, 45 & 66), determined by a series of 4 PCR of blood samples to be *A. phagocytophilum*-free, were used for these studies ad was similar to the previously reported study by Kocan et al. (2012). Sheep 44 and 45 were experimentally infected by intravenous inoculation (IV) of HL-60 cell cultures infected with the human NY-18 isolate of *A. phagocytophilum*, and Sheep 66, not inoculated with infected cell cultures, served as the uninfected control. Ticks were placed in cloth stockinettes on the sheep and allowed to feed in cells 1 and 2 for 2 or 4 days, while cell 3 remained without ticks. Skin biopsies were collected beneath tick feeding and non-feeding sites immediately after the sheep were euthanized for histopathology, IHC and PCR studies.

Animals and Sampling

Ticks

Ixodes scapularis ticks (180 male and female pairs) were obtained from the laboratory colony maintained at the Oklahoma State University Tick Rearing Facility. Adult ticks

were raised at the Tick Rearing Facility and the male and female ticks were then purchased and used for these studies. Off-host ticks were maintained in a 12 h light: 12 h dark photoperiod at 22–25°C and in humidity chambers with a 95% relative humidity (RH).

Sheep

Three Suffolk female sheep, approximately 1 year old, were originally purchased by the Tick Rearing Facility at a local livestock auction and subsequently bought by our laboratory for use in these studies (Fig 1; Sheep 44, 45 and 66). The sheep were determined to be negative for *A. phagocytophilum* by PCR analysis of the initial four blood samples that were collected twice per week. Sheep 44 and 45 were then inoculated IV with HL-60 cell cultures infected with *A. phagocytophilum* and Sheep 66 was not inoculated with cell cultures and was a source of uninfected ticks and sheep tissues as described previously by Kocan et al. (2013). All sheep were observed daily for clinical signs of disease. Blood samples were collected 2 times per week from all sheep and stained blood films were prepared from EDTA-anticoagulated blood on each collection day and stained with an aqueous Romanowsky stain using an automated stainer (Aerospray 7120, Wescor Inc., Logan, UT, USA). The stained smears were then examined for the presence of characteristic *A. phagocytophilum* morulae in granulocytes. All blood samples were also stored at -20 C and then tested for *A. phagocytophilum msp4* PCR assay to confirm that the seep remained infected throughout the study.

***A. phagocytophilum* propagation in HL-60 cells and infection of sheep**

The human NY-18 isolate of *A. phagocytophilum* (Asanovich et al. 1997; de la Fuente et al. 2006) was propagated in cultures of the human undifferentiated HL-60 promyelocytic cell line. Infected and uninfected cell cultures were maintained at 37°C in RPMI medium as reported previously (de la Fuente et al. 2005a). Sheep 44 and 45 were inoculated with 2 T-25 flasks of *A. phagocytophilum*-infected HL-60 cells (45% infection, as determined by detection of intracellular morulae in stained cytospin smears; Hema-3 Stain, Fisher Scientific, Middletown, VA, U.S.A.). The cultures were centrifuged and re-suspended with serum-free RPMI 1640 medium for a final dose of 1×10^7 cells in 2 ml of cell culture medium.

Inoculation of sheep, tick infestations and collection of ticks and skin biopsies

After Sheep 44 and 45 were determined by PCR to be infected with *A. phagocytophilum* they were used, along with uninfected Sheep 66, for tick feeding studies. The sheep were infested with ticks at one month after the sheep were determined as PCR positive for infection with *A. phagocytophilum*. This 30 day time period was found in previous studies to be optimal for acquisition of *A. phagocytophilum* infections by ticks (unpublished results). For tick feeding, three tick feeding cells (Cells 1, 2 and 3), made from 8 inch cotton orthopedic stockinettes (Tex-Care Medical, Burlington, N.C., U.S.A.), were glued to a shaved and washed areas on the sides of the sheep using industrial adhesive (#M Scotch-Grip Industrial Adhesive 4799, 3M Industrial Adhesvies and Tapes, St. Paul, MN U.S.A.). Cells 1 and 2 were placed 8 inches apart on the upper left side on the sheep and Cell 3, which was not infested with ticks, was placed on the upper right side of the sheep. Twenty male/female pairs of *I. scapularis* were then placed in each cell and allowed to feed. Cells 1 (4-day fed ticks) and 2 (2-day fed ticks) were infested with ticks on Day 0 and Day 2, respectively. Cell 3 did not receive ticks and served as a site

for collection of tick free (control) skin biopsies for histopathology and ICH studies. The tick feeding times (2 and 4 days) were based on previous studies in which advanced necrosis was observed in longer tick feeding times which would not be favorable for histologic and IHC studies (Kocan et al. 2010 and unpublished results). On Day 4, all ticks were removed, and the feeding lesions were photographed and marked for biopsy collection (Fig. 2c). After collection of blood samples, the three sheep were then euthanized by a licensed veterinarian with 1 mL / 10 lbs body weight of pentobarbital in the vein. Immediately after being euthanized three 8 mm full thickness punch skin biopsies of selected feeding and non-feeding sites were taken from each of the three cells on each sheep and placed in buffered formalin for histopathology, immunohistochemistry (IHC) or in Tri-reagent for PCR studies. The three skin biopsies taken from each cell were processed as follows: (1) one skin biopsy was fixed in buffered formalin for paraffin embedment. Sections prepared from these tissue blocks were either stained with hematoxylin and eosin (H&E) for characterization of the histopathology of the feeding lesions or left unstained for IHC studies, (2) one skin biopsy was homogenized and used for extraction of RNA and DNA for PCR and gene expression studies and (3) one skin biopsy from each cell was fixed in glutaraldehyde and stored for future light and electron microscopy studies. Ten of the female ticks collected from each cell were cut in half separating the left and right sides. Tick guts and salivary glands were then dissected from ½ of each of 10 female ticks per cell (Cell 1, 4d fed ticks; Cell 2, 2d fed ticks) on infected Sheep 44 and 45 and uninfected Sheep 66, and placed in Tri-reagent for subsequent DNA and/or RNA extraction. The other ½ tick was placed in glutaraldehyde fixative and stored for future microscopy studies. Necropsies were performed on the

sheep for gross and histopathologic evaluation, and samples of skin, liver, lung, heart, lymph node, and kidney were fixed in buffered formalin for histopathology or placed in Tri-Reagent for PCR studies. Use of experimental sheep for this research was done under protocol VM1026 approved by the Oklahoma State University, Institutional Care and Use of Animals Committee according to the regulations of the U.S. Department of Agriculture.

Detection of *A. phagocytophilum* by PCR in sheep and tick tissues

For PCR studies, DNA was extracted from tissue samples in Tri-Reagent (Sigma, St. Louis, MO, U.S.A.) following manufacturer's recommendations. Blood samples tested by PCR were collected from sheep prior to inoculation with *A. phagocytophilum* infected HL-60 cells, then twice per week post inoculation and immediately prior to euthanasia. Skin biopsies for PCR studies were collected immediate after euthanasia. Other sheep tissues tested by PCR were collected at necropsy and included spleen, liver, heart, skin, lung and cervical and mesenteric lymph nodes. *A. phagocytophilum* infection levels in sheep and tick tissues were characterized by *msp4* PCR on DNA samples as described previously by Kocan et al. (2010) using the Applied Biosystem 2720 Thermocycler as described previously (Busby et al. 2011) and using oligonucleotide primers MSP4-L (5' -CCTTGGCTGCAGCACCACCTG-3') and MSP4-R (5' TGCTGTGGGTCGTGACGCG-3'). PCR products were analyzed by agarose gel electrophoresis to identify positive samples. To corroborate the identity of the *A. phagocytophilum* isolate, *msp4* amplicons were resin purified (Wizard; Promega) and cloned into pGEM-T vector (Promega) for sequencing (Core Sequencing Facility, Dept.

of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University).

Real-time quantitative PCR

To quantify infection levels in each sheep tissue by real time PCR, 100 ng of total DNA was used in a 20 µl reaction, using the primers for *msp*4 PCR described in the previous section and the SsoAdvanced SYBR Green Supermix (Biorad Hercules, CA, U.S.A.). The *Ovis aries aldolase B* (GenBank accession number NM_001009809.1) was amplified using primers Oa-ALDOBF (5' -CCCATCTTGCTATCCAGGAA -3') and Oa-ALDOBR (5'-TACAGCAGCCAGGACCTTCT -3') and used for normalization. The reaction mixes were placed in the CFX96 Thermal cycler (BioRad Hercules, CA, U.S.A.). The cycling program for both PCRs consisted of 1 cycle of denaturation at 98ºC for 2 min and 40 cycles of annealing-extension at 55ºC for 1 min. and melting curve 55-95 ºC in 0.5 ºC increments 5 sec/step. Each run contained two negative controls with no DNA added. Triplicate values for infected and uninfected samples were normalized by calculating the ratio of *A. phagocytophilum msp4* product to averaged *Ovis aries aldolase B* product. Normalized values were averaged and the standard error of the mean was determined. The data were statistically analyzed using the Student's t-test (P=0.05).

Microscopic examination and assessment of inflammation

Skin biopsies fixed in buffered formalin were trimmed to the lesion sites then processed for paraffin embedment. Sections (3 µm) were prepared and stained with H&E for histologic evaluation. The stained slides were scanned and analyzed using the Spectrum 11.1.1.760 Software (Aperio Technologies Copyright 2006-2011, Vista, CA USA). Three separate areas (100 µm x 100 µm) were selected at 40 X magnification on biopsy

sections from Cells 1-3 on Sheep 44, 45 and 66 and the eosinophils and neutrophils cells within each area were counted and tabulated. These data were then statistically analyzed using the Student's t-test (P=0.05).

Immunohistochemistry

MSP4 antibody production

Recombinant *A. phagocytophilum* NY18 MSP4 (AFD54597) protein was expressed in *Escherichia coli* (Champion pET 101 Directional TOPO Expression kit, Carlsbad, CA, U.S.A.) and purified using the Ni-NTA affinity column chromatography system (Qiagen Inc., Valencia, CA, U.S.A.) following manufacturer's recommendations and as described by Ayllón et al. (2013). Rabbits were then immunized with purified MSP4 and serum samples were collected from the pre-immune and immunized rabbits from which the IgG was purified using Montage Antibody Purification Kit and Spin Columns with PROSEP-A Media (Millipore, Billerica, MA, U.S.A.), and stored frozen at -20^{o}C until used for the IHC studies.

Peroxidase-antiperoxidase (PAP) immunohistochemistry studies

Immunohistochemistry studies using peroxidase-anti-peroxidase (PAP) labeling on paraffin sections of skin biopsies from the infected and control sheep were done using the Vectastain Elite Rabbit IgG ABC Kit and AEC peroxidase substrate kit (Vector Labs, Burlingame, CA, U.S.A.). The PAP labeling reactions were done according to the manufacturer's instructions using *A. phagocytophilum* rabbit anti-MSP4 antibody prepared as described above. Control labeling reactions were done with MSP4 antibody on skin biopsy sections from the uninfected Sheep 66 and by using pre-immune antisera as the primary antibody in labeling reactions on biopsy sections from infected sheep. The

stained slides were examined and photographed using a Nikon Eclipse E600 and a Nikon DS-F12 camera with NIS Elements F4.00.00 software.

Confocal microscopy

Rabbit antibodies against *A. phagocytophilum* MSP4 were used for confocal microscopy studies on biopsy sections from infected and uninfected sheep. The sections were placed in xylene to remove the paraffin, and rehydrated in decreasing concentrations of ethanol followed by enzymatic antigen retrieval using proteinase K (Dako North America, CA, U.S.A.) (40 µl in 2 mL phosphate buffered saline [PBS]) for 7 min at room temperature, after which the slides were washed in PBS and blocked by incubation with 2% bovine serum albumin (BSA) in PBS at room temperature for 60 minutes. The primary MSP4 antibody (100 µl) diluted with buffer (2%BSA + PBS) ratio of 1:100 was then applied to sections and allowed to incubate at overnight at 4ºC. FITC conjugated anti-rabbit IgG antibody (Sigma-Aldrich, MO, U.S.A.) diluted in PBS+2%BSA at a ratio of 1:160 was used as the secondary antibody and the slides were allowed to incubate for 60 min in the dark at room temperature. The slides were then rinsed with PBS + Tween followed by a second wash of PBS. DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich, MO, U.S.A.) was prepared by adding 0.5 µL of DAPI to 5 mL of PBS and incubated at 37°C for 15 minutes, after which the slides were rinsed with PBS and dried. A single drop of anti-fade reagent (Invitrogen Molecular Probes, OR, U.S.A.) was placed directly over each sample followed by application of a glass coverslip and the slides were sealed. The slides were examined and photographed at 40x using a Leica SP2 Laser Scanning Confocal Microscope located in the OSU Electron Microscopy Laboratory.

Gene expression analysis by real-time RT-PCR

Immune response genes studied in blood and biopsy samples for this study were selected based on previous studies of blood samples from sheep experimentally infected with *A. phagocytophilum* (Galindo et al. 2008; Table 2). The analysis of gene expression in blood samples from infected and control sheep included erythropoeitin receptor (EPO-R), tight junction protein 2 (Z02), tight junction protein 3 (Z03), G-protein linked receptor (EDG2), interleukin 2RA (IL-2RA), toll-like receptor 7 (TLR7), desmoglein (DSG), macrophage migration inhibition factor (MIF), integrin alpha 4 (INTEG4; CD49d), G protein-coupled receptor (CCR7), and monocyte chemo-attractant protein 1 receptor MCP-1RA (CCR2). Gene expression was also studied in skin biopsies from the infected and control sheep and included EDG-2, IL-4RA (interleukin 4RA), CCR7, EPO-R, TLR7, DSG, CCR2, and ZO3. Total RNA was isolated from blood and skin biopsies samples of infected and uninfected sheep using Tri-Reagent (Sigma) and following manufacturer's recommendations. The primers for each of the genes and the real time RT-PCR procedures were as reported previously by Galindo et al. (2008). The iScript One-Step RT-PCR Kit with SYBR Green QuantiTec SYBR Green RT-PCR kit (Qiagen) and the CFX96 Thermal cycler (Bio-Rad Hercules, CA, U.S.A.) were used following manufacturer's recommendations. The mRNA levels were normalized against sheep beta actin as described previously (Galindo et al. 2008). In order to analyze gene expression in response to *A. phagocytophilum* infection in blood, samples from the same sheep before (Day 0) and after (Day 33) infection were used to avoid animal-to-animal variation. The expression of immune response genes in skin biopsies was analyzed using three different comparisons: (1) skin biopsies from feeding tick sites versus skin biopsies from sites without ticks in infected and uninfected sheep, (2) skin biopsies in sites

without feeding ticks in uninfected and infected sheep, and (3) skin biopsies with feeding ticks at the bite site in uninfected and infected sheep. Statistical analysis was conducted using Student's t-test with unequal variance (P = 0.05) for skin biopsies and by ANOVA test (P = 0.05) for blood samples.

Results

PCR detection of *A. phagocytophilum* in sheep tissues and ticks

Sheep. Sheep 44 and 45 were determined by PCR to be infected with *A. phagocytophilum*at 12 and 15 days post inoculation (PI), respectively, and blood samples collected throughout tick feeding and tested as a group after the end of the study confirmed that the sheep remained infected with *A. phagocytophilum*. Sequence analysis of the *msp4* amplicons from PCR products confirmed that both sheep were infected with the NY-18 strain of *A. phagocytophilum* used to initiate infection. Samples of blood, spleen, heart, liver, lung, skin and cervical and mesenteric lymph nodes collected at necropsy from the infected sheep were also PCR positive for *A. phagocytophilum*, with the exception of heart tissue from Sheep 45 which was negative. All blood and tissue samples collected from Sheep 66 throughout the experiment were confirmed by PCR to be negative for *A. phagocytophilum*.

Ticks. Salivary glands and guts (90% to 100%, respectively) from ticks that fed on infected Sheep 44 and Sheep 45 for 2 and 4 days were PCR positive for *A. phagocytophilum* (Table 1). Salivary glands and guts from ticks that fed on the uninfected Sheep 66 were PCR negative for *A. phagocytophilum*.

Quantitative PCR on sheep tissues collected at necropsy.

Significant differences were not observed by quantitative PCR in infection levels of *A. phagocytophilum* in spleen, liver, heart, skin, lung, cervical and mesenteric lymph nodes tissues, skin biopsies and blood samples collected at the time of euthanasia.

Necropsy findings.

Relevant gross changes seen at necropsy were similar in both infected sheep (44 and 45) and were mild and restricted to the lymphoid system. While the use of barbituates may have contributed to the mild splenomegaly that was observed, the lesions observed at necropsy would not have been impacted. Visible on the cut surface spleens of infected sheep were mildly enlarged with conspicuous lymphoid follicles. Sublumbar and prescapular lymph nodes were mildly to moderately enlarged, with maintenance of distinct corticomedullary architecture. In each of the sheep, gross lesions interpreted to be background changes unrelated to *A. phagocytophilum* infection were occasionally seen such as mild chronic pericardial adhesions with focal myocardial degeneration and fibrosis in sheep 45. Microscopic examination of lymphoid tissues revealed moderate lymphoid hyperplasia with germinal center formation in spleen and lymph nodes of each sheep. Moderate numbers of eosinophils and neutrophils were present in medullary sinuses of lymph nodes of both sheep and medullary cords were moderately expanded by increased numbers of plasma cells.

Histopathology of tick feeding lesions

Skin lesions were seen around feeding ticks (Fig 2a) and three feeding sites were marked after tick removal for biopsy collection (Fig 2, b and c). When biopsy sections stained with hematoxylin and eosin were examined with light microscopy, inflammation was seen in the 2 day tick feeding site biopsies all sheep (Fig. 3, Cell 2 column: 44-2,45-2 and 66-2) and after 4 days inflammation at tick feeding sites was pronounced in all sheep and

accompanied by necrosis (Fig. 3, Cell 1 column: 44-1,45-1, 66-1). Mild inflammation was also observed in the biopsies from Cell 3 of each sheep (Fig 3. Cell 3 column: 44-3, 45-3 66-3) where ticks had not fed. In the 4-day tick feeding biopsies from all sheep (Fig 3: Cell 1 column: 44-1, 45-1 and 66-1), the epidermis and underlying dermis were focally interrupted by an ulcer overlain by intense eosinophilic, neutrophilic and histiocytic inflammation within the dermis. Inflammatory cells were accompanied by abundant cellular debris within a background of edema. Dermal inflammation was most intense surrounding blood vessels and adnexal structures. Inflammation was less severe in biopsies from all sheep where the ticks had fed for 2 days (Fig. 3, Cell 2 column, 44-2, 45-2 and 66-2) and was restricted to the superficial dermis, in which a moderate inflammatory infiltrate composed of eosinophils and macrophages with fewer lymphocytes and plasma cells surrounded blood vessels and adnexa.

Analysis of the distribution of granulocytes in skin biopsies.

Analysis of the distribution of granulocytes in skin biopsies is depicted in Figure 4. The populations of eosinophils and neutrophils in skin biopsies from tick feeding sites were determined from the sections of the 2-day tick feeding sites (Cell 2) and the non-tick feeding sites of Cell 3 because inflammation and necrosis observed in 4-day tick feeding sites (Cell 1) was too advanced for accurate cell counts. The overall populations of granulocytes in the skin biopsies were significantly higher in biopsies from tick feeding sites (Cell 2) on all three sheep (44, 45 & 66) as compared with the non-tick feeding sites (Cell 3) (Fig. 4). The number of eosinophils was significantly higher in tick feeding sites in both infected and uninfected sheep ($P<0.03$; $N=3$). Neutrophils counts were significantly higher in biopsies from non-tick feeding sites in infected sheep when compared to the uninfected sheep ($P<0.05$; $N=3$). The number of neutrophils was higher in the infected

and uninfected sheep at tick feeding sites when compared to non-tick feeding sites (P=0.002; N=3). Total cell counts were higher in tick feeding sites in both infected and uninfected sheep, reflecting results of eosinophils counts (P<0.02; N=3). Additionally, total cell counts were higher in non-tick feeding sites of infected sheep 44 and 45 when compared to similar sites in the uninfected sheep 66 (P=0.004; N=3). These results suggested that eosinophils accumulate in tick feeding sites independently of infection, while neutrophils are associated with *A. phagocytophilum* infection.

IHC studies.

At low magnification focal fluorescence was not seen in sections of skin biopsies from the uninfected control sheep 66 (Fig. 5a). In contrast, infected cells were observed at low magnification in skin biopsy sections from the infected sheep that were taken from the 2-day tick feeding site (Cell 2; Fig. 5b) and the 4-day tick feeding site (Cell 1; Fig5c). At higher magnification IHC studies, infected neutrophils were localized by PAP labeled *A. phagocytophilum* MSP4 rabbit antibody in skin biopsies from infected sheep 44 and 45 cells in which ticks were allowed to feed Fig. 6a). PAP labeled cells were not seen in skin biopsy sections from the uninfected control (Sheep 66) or in biopsies from infected sheep reacted with pre-immune rabbit sera as the primary antibody. Likewise, infected cells were identified by confocal microscopy and indirect fluorescent antibody labeling in skin biopsies from infected Sheep 44 and 45 (Fig. 6, b and c). The nuclei of the infected cells were more clearly visible in sections that were counterstained DAPI (Fig. 6c).

Analysis of the expression of selected immune response genes

Expression of selected immune response genes in blood samples of infected sheep was not different from the uninfected sheep with the exception of Z02, which was significantly upregulated by 3- to 4-fold in the two infected sheep. In the analysis of

gene expression in skin biopsies from tick feeding sites versus skin biopsies where ticks had not fed, four genes (TLR, DSG, CCR2 and Z03) were significantly downregulated in response to tick feeding in Sheep 44, while Z03 was the only gene significantly downregulated in Sheep 45 (Table 2). Expression of immune response genes was not significantly different in skin biopsies in sites without feeding ticks in uninfected and infected sheep and in skin biopsies with feeding ticks at the bite site in uninfected and infected sheep (data not shown).

Discussion

This research is a continuation of our efforts toward development of a sheep model for the human NY-18 isolate of *A. phagocytophilum* for study of host/pathogen/tick interactions. Since our laboratory is most interested in tick-pathogen interactions, we chose sheep for this model because they are a good host for feeding large numbers of ticks required for gene expression and developmental studies. Previous studies have shown that genes are differentially expressed in sheep and tick cells in response to *A. phagocytophilum* (Galindo et al. 2008; Villar et al, 2010; Busby et al. 2012; Ayllón et al. 2013; Naranjo et al., 2013). In this research we also demonstrated differential expression of immune response genes in blood (upregulation of Z02 in response to infection) and skin biopsies from tick feeding sites of the experimentally infected sheep (downregulation of TLR, DSG, CCRS and Z03 in response to tick feeding). A similar study in mice demonstrated that tick feeding inhibits gene transcription and Th17 immunity in skin (Heinze et al. 2012). These results suggested that tick feeding downregulates immune response genes, thus contributing to mechanisms by which ticks counteract host immune responses (Hajdušek et al. 2013).

In our initial experiments (Kocan et al. 2012) we demonstrated by tick acquisition feeding studies that ticks are susceptible to infection with the NY-18 human isolate of *A. phagocytophilum* which was confirmed again by the results of this study. While *A. phagocytophilum* has been reported microscopically in tick gut muscle cells (Reichard et al. 2009), the complete description of tick developmental cycle of this pathogen is not known. Notably, even in the absence of clinical signs, these sheep served as an efficient host for infection of ticks (Kocan et al. 2010). This result led to our interest in further studying host-pathogen-tick inactions by characterizing the tick feeding site and the immediate environment where ticks acquire *A. phagocytophilum* infection. While infected granulocytes, as determined by the presence of morula, were not seen in blood smears of these experimentally infected sheep, IHC provided evidence of the multiplication of *A. phagocytophilum* in granulocytes at the tick feeding site which is likely to be the source of infection for ticks.

The necropsy results in this study were consistent with systemic inflammation and immune stimulation characterized predominately by hyperplasia of lymphoid tissues including splenomegaly which is commonly reported in *Anaplasma* infections (Blas-Machado et al. 2007; Kocan et al. 2003). By Day 2, tick feeding resulted in a dermal inflammatory response in which granulocytes, primarily neutrophils and eosinophils, were attracted to the tick feeding site, and by 4 days of tick feeding, inflammation was advanced with necrosis. These results are not surprising because ticks secrete a variety of salivary compounds during feeding that allow them to complete their blood meal despite the host immune response. Such salivary compounds have also been found to promote pathogen transmission, infection and infiltration of inflammatory cells to the bite site

(Kazimírova et al. 2013). Additionally, some of biomolecules secreted in tick saliva cause activation of the host's innate immune response promoting the infiltration of neutrophils and eosinophils into the bite site. Tick saliva biomolecules were recently reported to have a chemotactic effect, and to inhibit neutrophil and eosinophil function (Guo et al. 2009).

While the host inflammatory response to the tick feeding site was anticipated in these studies, we were surprised to find a significantly greater diffuse dermal inflammation at non-tick feeding sites in these experimentally-infected sheep. This generalized dermal inflammation is likely to be a mechanism that has evolved which enhances the coordination between tick feeding and infected granulocytes which would favor infection of ticks.

Confocal and PAP IHC studies revealed *A. phagocytophilum* infected granulocytes in the skin biopsies at the tick bite sites. These findings confirm previous ones in which *A. phagocytophilum* was demonstrated in granulocytes at the bite site on naturally infected sheep (Granquist et al. 2010). In this previous study, the majority of the inflammatory cells described at the tick bite site were neutrophils and monocytes, but interestingly lacking in eosinophils. However, the results were obtained on naturally infected sheep and non-tick feeding bite sites were not examined for analysis of dermal inflammation.

In summary, this research further advanced our understanding of the host/tick/pathogen interactions of the human NY-18 isolate of *A. phagocytophilum*. While sheep served as a good source of infection for ticks, as in our previous studies clinical signs were not apparent in any of the experimentally infected sheep (Kocan et al. 2012). Likewise, evidence was presented that tick feeding modulates the host immune response to

infection by altering the cellular infiltrates at the bite site and gene expression. Granulocytes recruited to the tick bite site appear to be the source of infection for ticks, and the increased levels of dermal inflammation in the infected sheep that apparently resulted from *A. phagocytophilum* infection were likely to enhance acquisition of tick gut and salivary gland infections in the 2- and 4-day fed ticks. While tick feeding Cells 1 & 2 were placed eight inches apart on the same side of the sheep, eight inches apart, Cell 3 without ticks was located on the opposite site of the sheep.

This model therefore provides a means of producing infected ticks for future tick/pathogen developmental morphologic and molecular interaction studies. This sheep model will also be useful for study of the molecular interactions between *A. phagocytophilum* and granulocytes and to determine the basis for the absence of recognizable morula in circulating granulocytes. It is likely that differences in gene expression in granulocytes occur at the bite site from that of the circulating granulocytes. Future studies are needed to further elucidate the specific genes involved in the development of dermal inflammation and *A. phagocytophilum* infection at the tick bite site.

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Table 1. The percentage of guts and salivary glands from female *I. scapularis* infected with *Anaplasma phagocytophilum* as determined by PCR

	Sheep 44, Cell 1 (2-day fed)	Sheep 44, Cell 2 (4-day fed)	Sheep 45 Cell 1 (2-day fed)	Sheep 45 Cell 2 (4-day fed)
Salivary Glands	100% (10/10)	90% (9/10)	100% (10/10)	100% (10/10)
Guts	100% (10/10)	100% (10/10)	90% (9/10)	90% (9/10)

Table 2. Expression of selected immune response genes in sheep skin biopsies.

Genbank accession number	Gene symbol	Gene description	Gene expression Ratio[a]	
			Uninfected sheep	Infected sheep
U18405	EDG2	G-protein linked receptor	0.7 ± 0.3	1.3 ± 0.5
AF081273	IL4R	Interleukin 4RA	0.1 ± 0.2	1.6 ± 0.2
NM_001024930	CCR7	G protein-coupled receptor	0.1 ± 0.2	0.7 ± 0.5
AY029231	EPO-R	Erythropoietin receptor	0.1 ± 0.2	0.7 ± 0.2
EF583900	TLR7	Toll like receptor 7	0.2 ± 0.4*	1.3 ± 0.2
M58165	DSG	Desmoglein	0.2 ± 0.2*	0.8 ± 0.2
XM_584158	CCR2	Monocyte chemoattractant protein 1 receptor	0.2 ± 0.2*	1.4 ± 0.1
AJ313186	ZO3	Tight junction protein 3	0.1 ± 0.3*	0.4 ± 0.1*

[a]Genes were selected based on the research reported previously by Galindo et al. (2008). Normalized mRNA levels were represented as ratios (Ave ± S.D.) of biopsies from Cell 2, 2-day tick feeding sites over skin biopsies from Cell 3 without ticks and compared between samples by Student's t-test (*$P<0.05$; $N=3$).

Figure 1. Experimental design for the infection of sheep and ticks with *A. phagocytophilum* and for collection of skin biopsies from sheep at tick feeding and non-tick feeding sites.

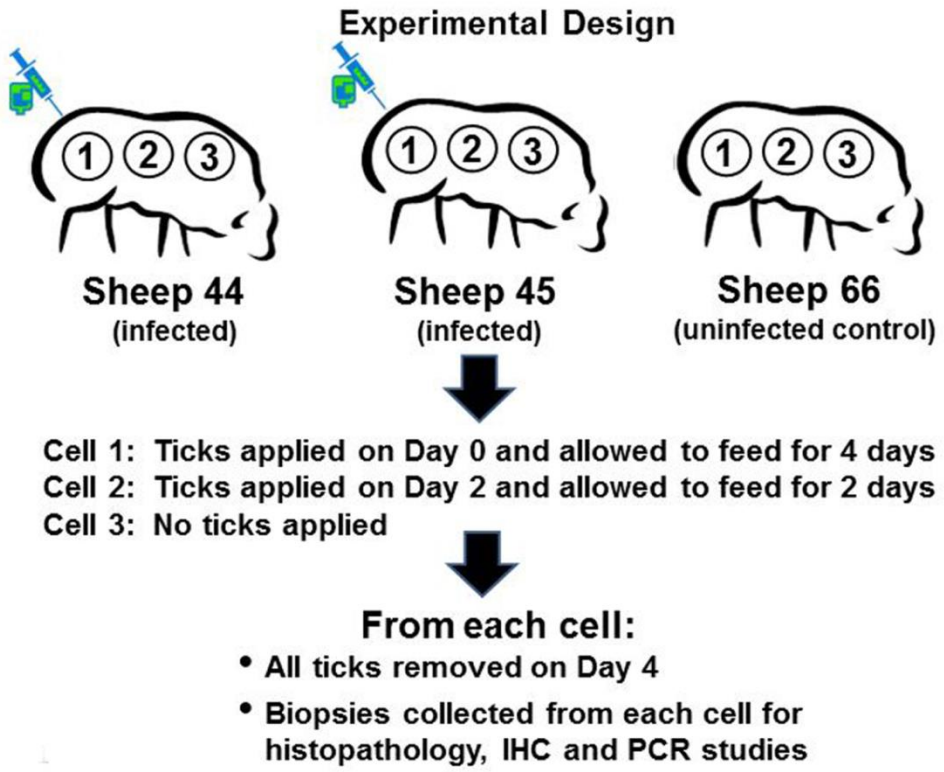


Figure 2. Tick feeding sites on sheep. (a) Lesions (arrows) around *I. scapularis* feeding sites on sheep; (b) The same skin lesions (arrows) after removal of ticks; and (c) three tick feeding lesion sites marked for collection of skin biopsies.

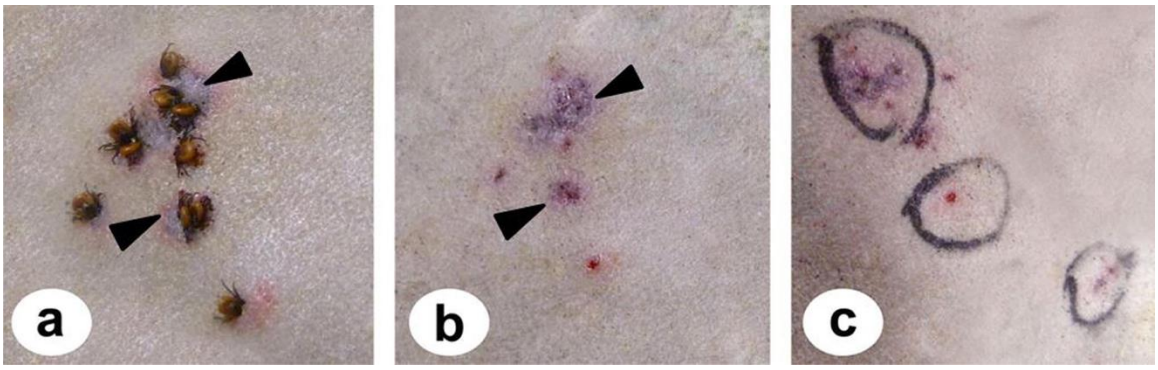


Figure 3. Light photomicrographs of hematoxylin and eosin stained sections of skin biopsies from infected Sheep 44 and 45 and uninfected Sheep 66. Column 1 sections of biopsies from Cell 2 after removal of the 2-day fed ticks (44-2, 45-2 and 66-2); Column 2- sections of biopsies from Cell 1 after removal of the 4-day fed ticks (44-1, 45-1 and 66-1); and Column 3-sections of biopsies from Cell 3 without ticks (44-3, 45-3 and 66-3). Bars = 100 µm.

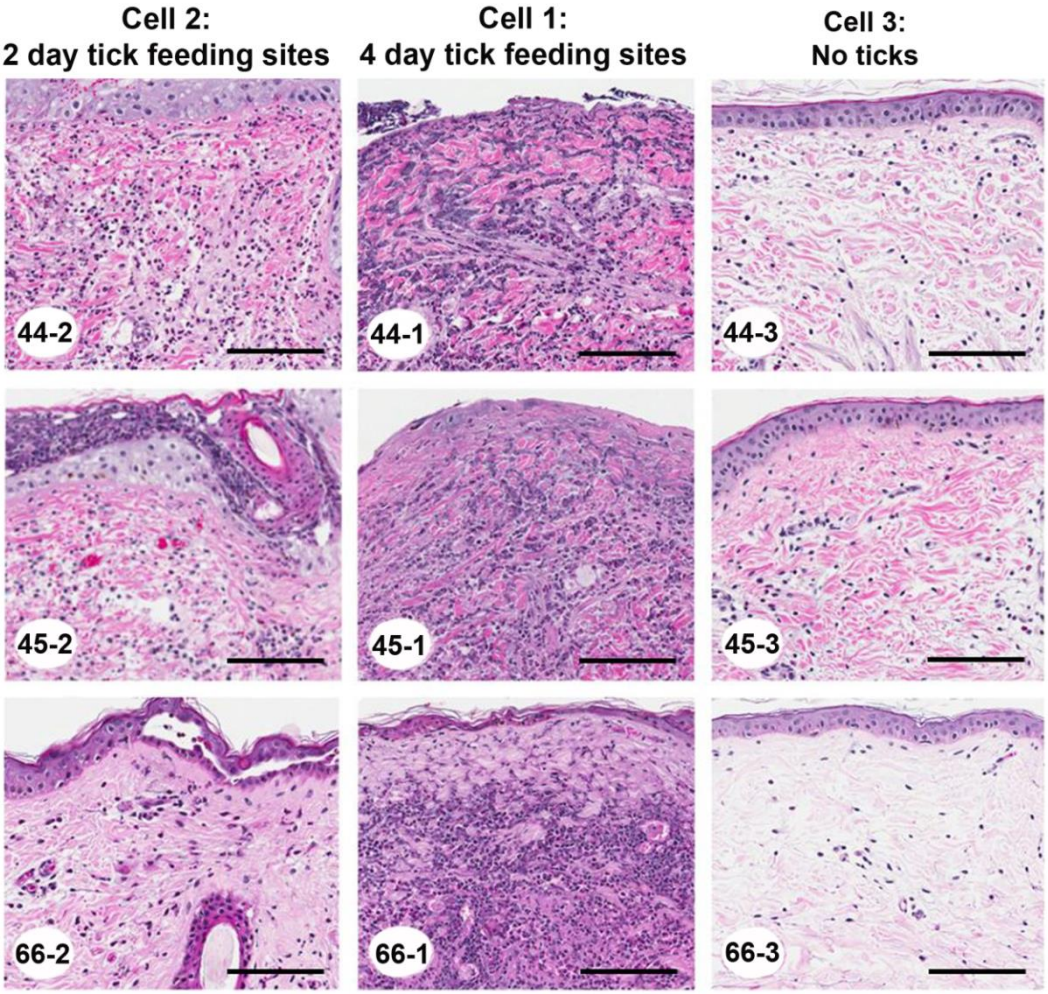


Figure 4. Graphic presentation of the distribution of eosinophils, neutrophils and the totals of both cell types in skin biopsies from infected Sheep 44 and Sheep 45 and uninfected Sheep 66. The data were analyzed using the Student's t-test (P=0.05).

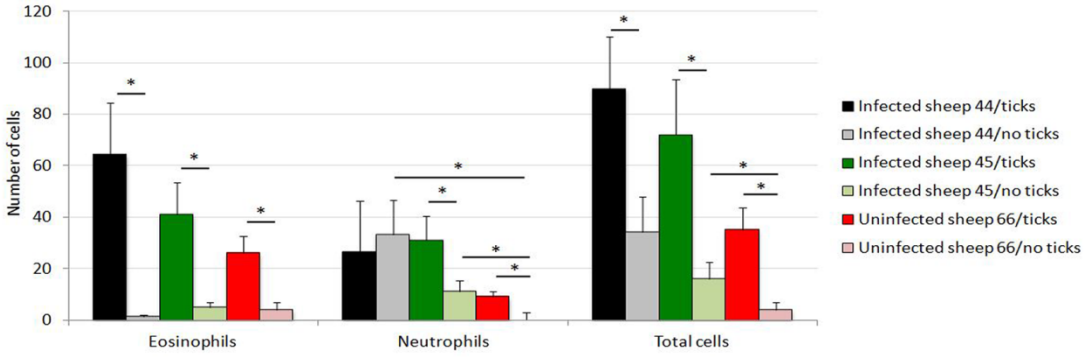


Figure 5. Low magnification light micrographs of the immunohistochemical localization of *A. phagocytophilum* infected neutrophils in sheep skin biopsies from Cell 2 in which the ticks had fed for 2 days. Rabbit polyclonal antibody against recombinant *A. phagocytophilum* MSP4 was used as the primary antibody. (a) uninfected sheep 66; (b) infected Sheep 44; and (c) infected Sheep 45. Cells infected with *A. phagocytophilum* seen in (b) and (c) appear as a bright punctate fluorescence. Bar = 100 µm.

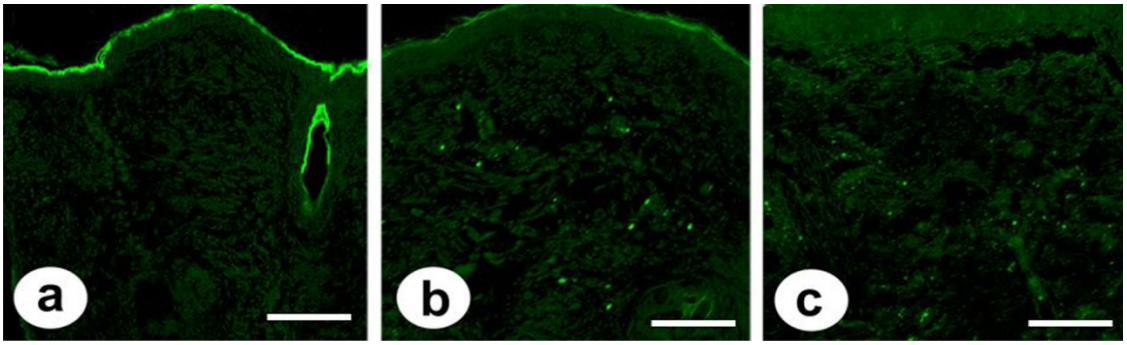
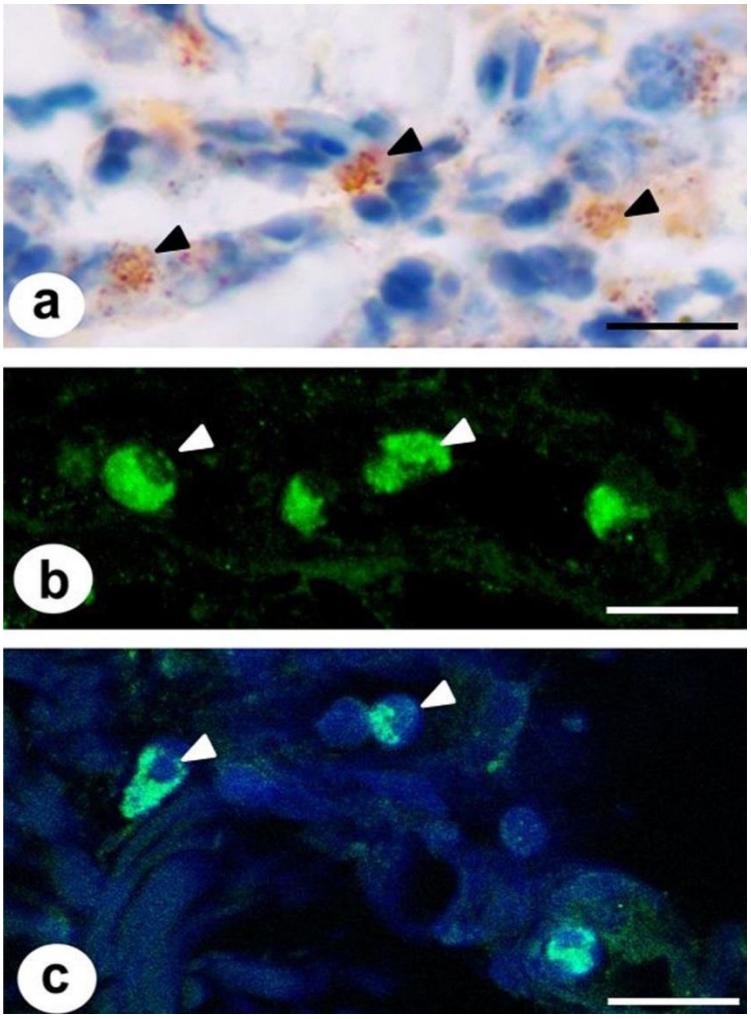


Figure 6. High magnification light micrographs of the immunohistochemical localization of *A. phagocytophilum* infected neutrophils in sheep skin biopsies using rabbit polyclonal antibody to MSP4 as the primary antibody. (a) *A. phagocytophilum* localized in neutrophils using perxoidase-antiperoxidase (PAP) conjugated to anti-rabbit IgG antibody (arrows); (b) and (c) *A. phagocytophilum* localized in neutrophils using confocal microscopy and FITC conjugated anti-rabbit IgG antibody 7(arrows). In (c) DAPI was used as a counter stain. Bar = 10 μm.



CHAPTER IV

SUMMARY

Anaplasma phagocytophilum, a gram negative obligate intracellular bacterium, has a tropism for granulocytes in the vertebrate host where it resides within a parasitophorous vacuole in the host cell cytoplasm. This tick-borne pathogen, first described in sheep in Europe as the etiologic agent of tick-borne fever (TBF), is transmitted primarily by ticks of the genus *Ixodes*. More recently, *A. phagocytophilum* has been shown to be the agent of granulocytic anaplasmosis in a variety of other species, including dogs, cats, llamas/alpacas, birds, rodents, deer, and has been recognized as an emerging tick-born disease of humans in the U.S. and Europe. The recognition of the broad distribution of *A. phagocytophilum* and its emergence as a human tick-borne pathogen, particularly in the U.S., have created renewed interest and resulted in accelerated research on this organism, particularly concerning its molecular relationship with vertebrate and tick hosts. Recently, variants of *A. phagocytophilum* isolated from humans with HGA were found by sequence analysis to differ from than those that cause disease in ruminants. Because *A. phagocytophilum* is infective for a wide range of mammals, this pathogen may emerge in other animal populations in the future, such as food animal species, which may impact food animal production and also pose risk of increased exposure of humans.

Research in our laboratory was directed toward development of a sheep model for studying host-pathogen-vector interactions and the NY-18 human isolate of *A. phagocytophilum*. Sheep were shown to be susceptible to infection with *A. phagocytophilum* and served as a host for infection of *I. scapularis* ticks (Kocan et al. 2010). The research presented in this thesis confirmed our initial findings and extended our understanding of this sheep model by characterizing the tick feeding sites on infected sheep and describing factors that may favor infection of the tick vector.

Ticks are the biological vector for *A. phagocytophilum* and are most often required for transmission between humans and their mammalian hosts. The developmental cycle of the organism within the tick has not been fully characterized, but infections of the Ap-1 variant of *A. phagocytophilum* have been demonstrated in tick gut cells. While recent research has contributed to advancing our understanding of the relationship between the tick, the host and the pathogen, further research is needed to elucidate the tick developmental cycle of *A. phagocytophilum* and to define the mechanisms of infction and transmission by ticks.

An unexpected result of this research was the confirmation of transplacental transmission of *A. phagocytophilum* in sheep. One of the two sheep used for this research was experimentally infected with the *A. phagocytophilum* organism during the third trimester of pregnancy. The sheep gave birth to a lamb that was depressed and not feeding and was subsequently euthanized 18 h after birth. Tissues (blood, spleen, heart, skin, cervical and mesenteric lymph nodes) collected at necropsy tested positive for *A. phagocytophilum* by PCR, and sequence analysis confirmed that the lamb was infected with the NY-18 isolate. Transplacental transmission may therefore also be a means of *A.*

phagocytophilum transmission and may likely contribute to the epidemiology of tick-borne fever in sheep and other mammals. This was the first report of transplacental transmission of the *A. phagocytophilum* in sheep and may be a source of infection for lambs that were raised on tick free pastures. Further research is needed to better understand the specific role of transplacental transmission in the transmission cycle of *A.phagocytophilum.*

The focus of this research was to characterize the feeding site on sheep where ticks acquire infection with *A. phagocytophilum.* The overall hypothesis for this research was that *A. phagocytophilum* infected inflammatory cells were attracted to and sequestered at tick feeding sites where they served as a source of infection for ticks. To test this hypothesis, sheep were first reconfirmed as a model host for infection of ticks with the human NY-18 isolate of *A. phagocytophilum.* Skin biopsies were then collected at tick feeding sites on infected and uninfected sheep for histopathology and immunohistochemistry studies. The predominant cell types at the tick feeding site were found to be neutrophils and eosinophils. In addition, increased inflammation was observed in the dermis of experimentally infected sheep where ticks had not fed. Thus, *A. phagocytophilum* infection resulted in a generalized dermal inflammation as compared with the uninfected control sheep and localized inflammation is compounded by tick feeding. Immunohistochemistry using anitbodies to recombinant *A. phagocytophilum* MSP4 was done by two methods, indirect fluorescent antibody and peroxidase-anti-peroxidase labeled antibody, and both methods resulted in localization of *A. phagocytophilum* infection in granulocytes at the tick feeding sites. These infected cells are likely to be the source of infection for ticks.

Collectively, the results of this research demonstrated that *A. phagocytophilum* infection and tick feeding contribute to inflammation and likely modulate the recruitment of infected granulocytes to tick feeding sites where they serve as a source of infection for ticks. Over all, these studies have contributed to our understanding of the *A. phagocytophilum* interactions with vertebrate and ticks hosts.

VITA

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